Limnol. Oceanogr., 52(1), 2007, 407–415

Sensitivity of herbivorous zooplankton to phosphorus-deficient diets: Testing stoichiometric theory and the growth rate hypothesis

Aloysio da S. Ferrão-Filho[1] *and Alan J. Tessier*[2]
Kellogg Biological Station, Department of Zoology, Michigan State University, Hickory Corners, Michigan 49060

William R. DeMott
Department of Biology, Indiana-Purdue University, Fort Wayne, Indiana 46805

Abstract

Stoichiometry and growth rate variation are related aspects of the ecology and evolution of consumer-resource interactions. In zooplankton, these concepts have been explored primarily in a few species of *Daphnia*. We used growth bioassays and changes in animal P content to quantify the sensitivity of four herbivorous cladoceran species to algal resources representing a gradient in C : P ratios ranging from 140 to 1,000, offered at both low and high food levels. Supplements of phosphate, *Synechococcus*, or P-sufficient algae were used to test for P, energy, and fatty acid limitation. Phosphorus assimilation was estimated by isotope techniques (^{32}P) to test the hypothesis of digestion resistance in P-limited algae. The cladoceran species differed in sensitivity to P deficiency at both low and high food levels, although sensitivity was less at low food. The P content of the two *Daphnia* species changed substantially along the C : P ratio gradient, contradicting the notion of strict homeostasis, whereas the two other cladoceran species showed tight P homeostasis. Consistent with stoichiometric theory, the two species with the highest P content were also more sensitive to P deficiency. Growth rate was related to P content across the four species at the high food level and at low C : P ratios, supporting the growth rate hypothesis. The addition of supplements to P deficient algae improved animal's growth rates, showing both P and energy limitation, but no evidence for fatty acid limitation. Lower assimilation efficiency for P-deficient algae suggests that digestion resistance can be a factor in the food quality of P-deficient resources.

Ecological stoichiometry deals with "the balance of energy and multiple chemical elements in ecological interactions" (Elser 2000). Several studies have shown that the C : N : P ratios of primary producers varies markedly in terrestrial, marine, and freshwater ecosystems, whereas herbivores show much less variation in elemental composition (Sterner and Hessen 1994; Elser et al. 2000*a*; Sterner 2000). According to the dominant paradigm, P is the main limiting element in freshwater systems, and seston C : P ratios suggestive of P limitation of herbivores (Sterner and Hessen 1994; Sterner 1997; Sterner and Elser 2002) are commonly found in lakes (Elser et al. 2000*a*; *but see* Brett et al. 2000). However, growing evidence suggests that herbivores are often limited by energy, even when seston is deficient in P as a result of the development of digestion-resistant phytoplankton (DeMott and Tessier 2002; DeMott et al. 2004*a*; Ferrão-Filho et al. 2005).

Two assumptions form the basis of stoichiometric theory: first, that consumers display tight elemental homeostasis (Sterner and Hessen 1994; Anderson and Hessen 1995; Sterner 1997), and second, that elemental limitation should occur only when food is above the threshold food concentration for growth (Sterner 1997). The logical explanation for the second assumption is that, at very low food levels, C requirements for growth are insufficient, and thus N and P are unnecessary for biomass construction; hence, C will be used only as energy for maintenance (Sterner and Hessen 1994; Sterner 1997). This model also assumes that P-deficient diets lead to consumer P excretion rate approaching zero and P assimilation efficiency approaching 100%. Thus, the only ways to maintain strict element homeostasis in P-deficient diets is to either excrete the excess C or to reduce C assimilation and to maximize the assimilation of phosphorus. However, DeMott et al. (1998) showed experimentally that the premise of both zero P excretion and 100% P assimilation efficiency were not satisfied, although they found that C assimilation efficiency was reduced in P-deficient diets.

By using experimental data from other authors, Anderson et al. (2005) modeled the effects of continued P excretion when consumers feed on P-deficient resources. They suggested that *Daphnia* could alter the balance of nutrients (C, N, and P) in their body by a "postabsorptive" process in the gut as a means of releasing the substrates in excess and reach relative element homeostasis. They also showed that this "stoichiometrically regulated release" is possible even in low food levels. Although this is still an area of controversy, some evidence shows that animals

[1] To whom correspondence should be addressed. Present address: Departamento de Biologia, Instituto Oswaldo Cruz, FIOCRUZ, Laboratório de Avaliação e Promoção da Saúde Ambiental, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ-CEP 21045-900 (aloysio@ioc.fiocruz.br).

[2] Present address: National Science Foundation, Division of Environmental Biology, Arlington, Virginia 22230.

Acknowledgments

We thank Pamela Woodruff for laboratory assistance and Dr. Michael Klug for the use of his laboratory during the isotope experiments. ASFF acknowledges support from FAPESP-01/04444-3, AJT acknowledges support from NSF-IOB 0235118, and WRD acknowledges support from NSF-IOB 0225474. This is contribution 1215 of the W. K. Kellogg Biological Station.

might also balance C by increasing their respiration rates (Sterner 1997; Darchambeau et al. 2003; Anderson et al. 2005).

The growth rate hypothesis (GRH), which states that P content is directly coupled to high demands for P-rich ribosomal RNA in fast-growing organisms, has been suggested as an adaptive explanation for variation in the P content in consumer taxa (reviewed by Elser et al. 2000*b*). According to the GRH, organisms with high P demands should possess the highest growth rates under P-rich food conditions, but should also be more sensitive to resource P limitation. The prediction is that, across taxa, maximum growth rates should increase with P content (Elser et al. 2003). However, this relationship may disappear or even reverse under P limitation.

Although several studies have examined the consequences of dietary P deficiency at moderate to high food levels, few studies have examined P limitation in cladocerans at low food levels (Sterner and Robinson 1994; Boersma and Kreutzer 2002; Acharya et al. 2004). Further, these studies have all focused on a single genus, *Daphnia*, which has also restricted testing of the GRH. We sought to test the effects of P-deficient algae at both low and high food levels on the growth rate, P content, and P assimilation efficiency of four cladoceran species differing in body size and phylogenetic relatedness. We performed growth bioassays and manipulated the dietary P content of the food, creating a C : P ratio gradient, and analyzed cladoceran sensitivity to P limitation by means of a regression technique (Tessier and Woodruff 2002). We also used a supplementation approach (DeMott 1998) to study the response of cladocerans to supplements of phosphate, *Synechococcus* (a P-rich cyanobacterium but poor in essential fatty acids and sterols; Brett and Müller-Navarra 1997; Von Elert 2002) and P-sufficient green alga.

Material and methods

Culture of animals—Four cladocerans species were cultured in the laboratory with P-free zooplankton artificial medium (ZAM; Tollrian 1993) to minimize changes in resource P content during the experiments. A hybrid clone of *Daphnia pulex-pulicaria* (hereafter *D. pulex*) was obtained from cultures maintained at the W. K. Kellogg Biological Station, Michigan State University, and all other clones (*Ceriodaphnia richardi, Daphnia ambigua*, and *Moina micrura*) were obtained from the cultures maintained at Universidade de São Paulo, which originated from the shallow, mesotrophic Lake Monte Alegre (São Paulo, Brazil). All clones were fed 0.5 mg C L^{-1} of *Ankistrodesmus falcatus*, a high-quality green alga (molar C : P ~137).

Growth bioassays—Two kinds of experiments were performed in order to quantify the ability of the four species to grow when fed P-limited and digestion-resistant foods. First, juveniles (<24 h) were grown in food treatments representing a gradient of C : P ratios created by mixing P-sufficient algae (C : P ~ 140) and P-deficient algae (C : P ~ 1,000). For culturing P-deficient algae, about 5 mL of a P-sufficient *A. falcatus* culture were inoculated in 100 mL of MBL medium lacking phosphate (as in DeMott 2003). This culture was grown for 5–7 d at continuous light on a shaker (~1 rpm). Mixing P-sufficient and P-deficient algae in the proportions of 1 : 0, 1 : 1, 1 : 5, and 0 : 1, we obtained C : P ratios of 140, 250, 450, and 1,000, respectively. These growth bioassays were conducted in two food levels, low food (0.1 mg C L^{-1}) and high food (1.0 mg C L^{-1}), in order to measure how P limitation is affected by food level. Although the lower food level is above the threshold food concentrations for growth, energy limitation should be strong and might be expected to ameliorate P limitation.

In the second experiment, juveniles of each species were subjected to the following treatments: P-deficient green algae (P^-Ank); P^-Ank supplemented with 0.5 μmol L^{-1} of KH_2PO_4 (phosphate; $+PO_4$); P^-Ank supplemented with *Synechococcus* (+Syn), a cyanobacterium rich in P but poor in EFA; and P^-Ank supplemented with 0.5 μmol L^{-1} phosphate and P-sufficient green algae ($+PO_4$ + P^+Ank). In the second and fourth treatments, we incubated P^-Ank with added phosphate for 30 min before the start of the experiments. P-deficient algae have been shown to uptake nearly all phosphate at the experimental concentration within 15 min (Plath and Boersma 2001). In all treatments, food totalled 0.5 mg C L^{-1}, and food mixtures with two different carbon sources were always offered in the proportion of 1:1 (by mass).

Samples were collected from the food sources for measurement of C, N (Carlo-Elba C : H : N analyzer), and P (persulfate digestion followed by molybdate reaction). We calculated specific growth rate for each treatment and contrasts (differentials) between particular treatments as in DeMott et al. (2001). The growth rate differentials of the $+PO_4$ and +Syn treatments compared with the P^-Ank treatment (control) each provided a test of P limitation. The contrast between +Syn and $+PO_4$ treatments served as a negative control for limitation in essential fatty acids. The contrast between $+PO_4$ + P^+Ank and +Syn treatments tests whether animals can improve growth further even if satiated with P but with additional essential fatty acids provided by P^+Ank. If so, this would indicate a limitation in fatty acids or energy. It is important to note that *Synechococcus* and P-sufficient green algae are both readily assimilated sources of carbon (energy), and thus any improvement in growth with these supplements relative to $+PO_4$ treatment may indicate energy limitation as a result of digestion resistance in the P-deficient food.

Both experiments were conducted at 20°C, and started with 5 to 10 neonate animals <24 h old placed in beakers with 150 mL of algae suspensions in ZAM. There were two replicate beakers per treatment, and food was replaced every day. A random subsample of 10–30 neonates were harvested to get initial weights and assigned to P analysis. Juvenile growth rates were calculated according to the following equation: $g = (\ln W_t - \ln W_o)/t$, where W_o and W_t are the dry weights of juveniles at the beginning and after t days (t = 2 d for *Moina*, 3 d for *Ceriodaphnia*, and 4 d for *D. ambigua* and *D. pulex*). The last juvenile instar in *Moina* occurs very early (by day 3), while in most *Daphnia* species, it takes 4 or 5 d at this temperature. Thus, our experiments

were designed to finish before the first adult instar. Animals were dried at 55°C before weighing on a microbalance (Cahn, model C-31, Thermo Electron Corporation, Waltham, Massachusetts) to the nearest 0.1 μg. After weighing, animals were transferred to 10 mL volumetric flasks, ashed at 550°C, and analyzed for P as in DeMott et al. (1998).

Phosphorus assimilation experiment—To test the hypothesis that digestion resistance contributes to poor growth in P-deficient algae, two assimilation experiments were performed with P-sufficient and P-deficient algae (*A. falcatus*) by using the ^{32}P isotope method described in DeMott and Tessier (2002). A total of 100 mL of algae culture was labeled with 100 μL ^{32}P-phosphate (2 μCu mL^{-1}) and placed on a shaker (1 rpm) with continuous light the day before the experiment. We mixed 1.0 mL of labeled algae with 100 mL of unlabeled food and fed this to two to 3-d-old animals. There were four replicate beakers for each cladoceran species in each treatment, each beaker containing about 10 to 30 animals, depending on the size of the cladoceran species. Both experiments were conducted with phosphate-free ZAM to avoid contamination with sources of P other than food particles. In these experiments, either P-sufficient (C : P = 140) or P-deficient (C : P = 1,000) labeled algae was used at one food level (1.0 mg C L^{-1}). Each feeding trial started with the animals placed in a beaker with labeled algae. After 8 min of feeding, all animals were transferred to a beaker with ZAM without food and half of them to scintillation vials and rinsed with distilled water 3 times to remove any radioactive particles transferred together with the feeding solution. These animals were processed to estimate clearance rates. The other half was transferred to unlabeled alga (1.0 mg C L^{-1}) and allowed to clear their guts for 40 min. Assimilation efficiency was estimated from the ratio of the 40 min : 8 min net clearance rates (DeMott et al. 1998).

Radioactivity in the feeding solutions was estimated by filtering 2 mL through a 0.2-μm Nuclepore filter. Both the animals and the filters were digested in 0.5 mL of tissue solubilizer (TS-2, Research Products International, Mount Prospect, Illinois). Scintillation vials were filled with 10 mL fluor (Ecolume, ICN Radiochemicals, Irvine, California) and radioactivity was counted in a scintillation counter (Wallac 1409; Perkin-Elmer, Wellesley, Massachusetts).

Data analysis—Regression analyses were performed in order to estimate the dependence of juvenile growth rate on the C : P ratio of the food for each cladoceran species and each food level separately. The slope of the Model I regression (Systat, version 9; SPSS, Chicago, Ilinois) was used as a measure of species sensitivity (sensu Tessier and Woodruff 2002) to the C : P gradient. An ANCOVA was used to examine the relationship between growth rate and P content of the cladoceran species (as predicted by the GRH) and to test whether this relationship was affected by food level and C : P ratio (as categorical factors). One-way ANOVA was performed to detect differences in P contents of animals in both growth bioassays. ANOVA was also used to test for the effects of species and food P content on the assimilation efficiency of phosphorus. We used the homeostasis coefficient eta (H) to quantify changes in *Daphnia* P content relative to changes in resource P content (Sterner and Elser 2002). H is calculated by regressing the log of %P of animals versus the log of algal %P. H is the inverse of the slope of the linear regression and approaches infinity for perfect homeostasis and approaches 1.0 when a consumer's element content varies in direct proportion to the element content of its food.

Results

Both food level ($F_{1,32} = 315.88$; $p < 0.0001$) and C : P ratio ($F_{3,32} = 221.42$; $p < 0.0001$) had significant effects on growth rate, and there was also a significant interaction between these two aspects of food ($F_{3,32} = 16.27$; $p < 0.0001$). Animals showed negative responses proportional to the C : P ratio gradient at both low and high food levels (Fig. 1A,B). However, sensitivity was much greater in high food levels (Table 1). Sensitivity of the animals to the C : P gradient also varied among the species at both food levels (Table 1), but *D. ambigua* and *Moina* were always the most sensitive.

There was a significant effect of species ($F_{3,32} = 181.61$; $p < 0.0001$), food level ($F_{1,32} = 6.13$; $p < 0.0187$), and algal C : P ratio ($F_{3,32} = 38.88$; $p < 0.0001$) on the P content of cladocerans, and also a significant interaction between food level and C : P ratio ($F_{3,32} = 4.58$; $p < 0.0089$). Among the species, *D. ambigua* and *Moina* had the highest P content regardless of C : P ratio or food level. Hence, the two species with the greatest sensitivity of growth to P deficiency were also the two species with the highest P content. In general, the P contents of the animals declined from the beginning to the end of the growth period and reached lower values when resources were more P deficient (i.e., at high resource C : P ratios; Fig. 1C,D). *Ceriodaphnia* in both food levels and *Moina* in the low food level were exceptions to this trend. Consequently, the homeostasis coefficient (H) for P varied greatly among species, with *Ceriodaphnia* and *Moina* having the highest H values and *D. ambigua* and *D. pulex* having much lower values (Table 2). Animals showed a tendency toward higher H coefficients at low food levels, and this matches the lower sensitivity of growth to P deficiency at low food levels. But among species, there was no relationship between homeostasis of P and sensitivity of growth to P deficiency.

Growth rate was positively related to body P content among species but this relationship significantly varied with the food level and the resource C : P ratio (Figure 2; ANCOVA: P content × food level interaction $F_{1,54} = 19.2$, $p < 0.001$; P content × C : P ratio interaction $F_{3,54} = 555.4$, $p = 0.003$). At high food levels, growth rate increased greatly with P content of the species (Fig. 2A; slope = 0.328, $p < 0.001$) whereas at low food levels the relationship was not significant (slope = −0.05, $p = 0.44$). At low C : P ratios, growth rate increased with species P content, although only the lowest C : P ratio gave a slope significantly different from zero (Fig. 2B; C : P = 140, slope = 0.364, $p = 0.04$; C : P = 250, slope = 0.279, $p = 0.11$). At higher C : P ratios this relationship disappeared (C : P =

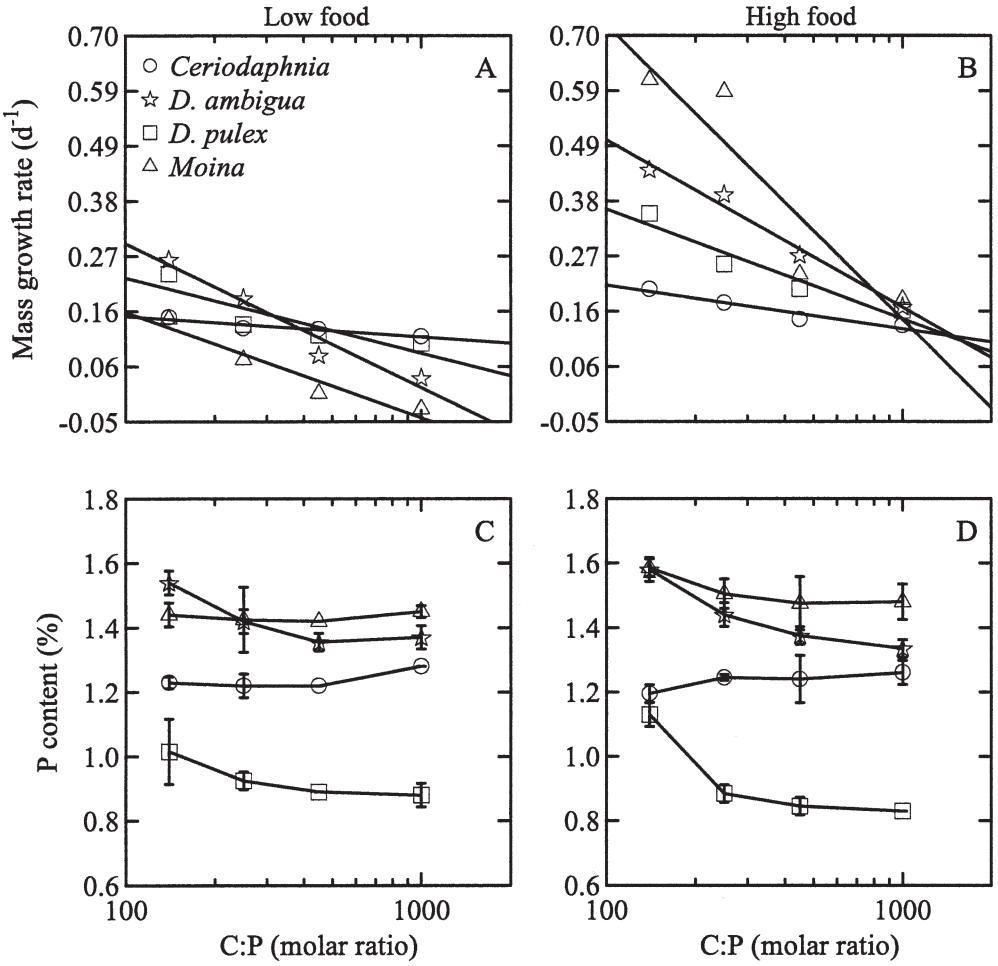

Fig. 1. First growth bioassay. (A, B) Growth responses of cladocerans to algal C : P gradient at low (0.1) and high (1.0 mg C L^{-1}) food levels. (C, D) Final P content of cladocerans after growth on C : P ratio gradient at low (0.1) and high (1.0 mg C L^{-1}) food levels. X-axes are in log scale. Error bars are standard deviation. Mean P-content values of juveniles at beginning of this experiment were as follows: low food: *D. ambigua* 1.60%, *D. pulex* 1.26%, *Ceriodaphnia* 1.38%, *Moina* 1.57%; high food: *D. ambigua* 1.63%, *D. pulex* 1.32%, *Ceriodaphnia* 1.41%, *Moina* 1.69%.

450, slope = −0.018, $p = 0.86$; C : P = 1,000, slope = −0.073, $p = 0.37$).

In the second experiment, animals responded positively to each food supplement. There was a significant effect of species ($F_{1,56} = 57.8$; $p < 0.0001$) and treatment (supplement; $F_{3,56} = 67.1$; $p < 0.0001$) and also a significant interaction between these factors on the growth rate of cladocerans ($F_{3,56} = 2.98$; $p = 0.0389$). Algae that were P deprived showed a moderate degree of P deficiency (Table 3; C : P~690), which was enough to reduce animal growth rate when offered as a sole food source. Addition of inorganic phosphate to P-deficient algae effectively reduced the C : P ratio to 360, promoting an increase in growth rate for both cladocerans (Contrast between P−Ank and $+PO_4$; *D. pulex*: $p = 0.0035$; *Moina*: $p = 0.0006$). Both cladocerans responded similarly to P limitation, as shown by the P growth rate differential (Fig. 3). Addition of *Synechococcus* (C : P~78) to P-deficient algae caused an even larger increase in growth rates (Contrast between $+PO_4$ and +Syn; *D. pulex*: $p = 0.0005$; *Moina*: $p < 0.0001$), suggesting that there was also energy limitation in addition to P limitation. However, the stronger response to the +Syn treatment may also have been due to the lower C : P ratio in that treatment compared with the phosphate addition treatment. Although *D. pulex* exhibited about the same response to energy addition as to phosphate addition, *Moina* showed a stronger response to energy limitation than to P limitation (Fig. 3). The addition of phosphate and P-sufficient algae, simultaneously, did not cause significantly increased growth rates relative to +Syn treatment, suggesting that a fatty acid deficiency was unlikely to have contributed to the poor growth with the P-deficient diet. Also, the larger increase in growth rate with *Synechococcus* (poor in EFA) by itself and the negative growth rate differential for "fatty acids" addition shown by both cladocerans suggest that these biochemicals were not important in limiting growth with P-deficient diets.

The P content of *Moina* did not change significantly with the different food supplements, but P content did change markedly in *D. pulex* ($F_{4,5} = 274.5$; $p < 0.0001$; post hoc

Table 1. Linear regression coefficients, p values, and sensitivity (slope) of cladocerans to the C : P ratio gradient in the first growth bioassay.

Cladoceran	n	r^2	p	Sensitivity (slope)
Low food				
Ceriodaphnia	8	0.364	0.113	0.04
D. ambigua	8	0.927	<0.001	0.28
D. pulex	8	0.738	0.006	0.15
Moina	8	0.888	<0.001	0.20
High food				
Ceriodaphnia	8	0.761	0.005	0.09
D. ambigua	8	0.951	<0.001	0.33
D. pulex	8	0.904	<0.001	0.21
Moina	8	0.834	0.002	0.57

Tukey test) (Fig. 4). There was a significant effect of species ($F_{1,10} = 211.2$; $p < 0.0001$) and treatment (supplement; $F_{4,10} = 30.8$; $p < 0.0001$) and also a significant interaction between these factors on the P content of cladocerans ($F_{4,10} = 29.7$; $p < 0.0001$). This result is similar to what was observed in the first experiment; *Moina* maintained a higher P content on average and had much tighter homeostasis compared with *D. pulex*.

In the phosphorus assimilation experiments, animals showed average assimilation efficiencies above 80% in all treatments, with lower assimilation efficiencies in P^-Ank ($F_{1,24} = 8.95$; $p = 0.006$) (Fig. 5). However, differences between treatments were significant only for *D. pulex* ($F_{1,6} = 7.52$; $p = 0.034$) and *Moina* ($F_{1,6} = 3.71$; $p = 0.010$).

Discussion

According to stoichiometry models, when carbon is at or below threshold food levels, energy limitation should preclude mineral limitation because carbon will be used only for metabolic maintenance (Sterner 1997). An assumption in these models is that, in spite of the high variability in food (algae) stoichiometry, herbivores are expected to maintain strict element homeostasis (Sterner and Hessen 1994; Sterner 1997; Sterner and Elser 2002). Our results support recent studies in demonstrating that consumer homeostasis is not strict and tightness of homeostasis varies with species (DeMott et al. 2004*b*; DeMott and Pape 2005). Our study species generally showed decreases in the P content of their tissues with decreases in the P content of their food in both low and high food levels, and three of the four species had quite large declines in P content with increased C : P ratio of their food. Despite this lack of homeostasis, we do find substantial support for the prediction that sensitivity to P limitation is reduced at low food concentrations.

Our data show a clear decrease in sensitivity to P deficiency when animals are raised at low compared with high food levels. Further, our low food level was high enough to produce relatively good growth for at least two of the four species, and a third showed no effect of P

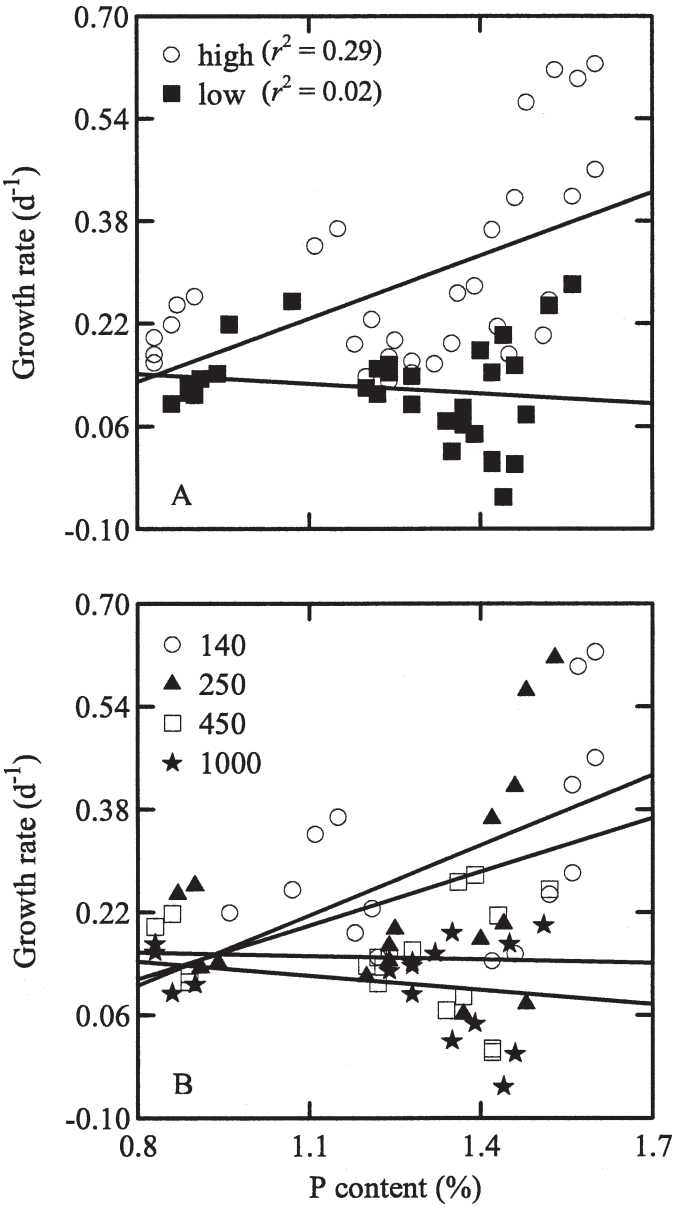

Fig. 2. Relationship between growth rate and P content of animals in first growth bioassay, grouped by (A) food level and (B) C : P ratio of food. Regression lines were fitted to data in each food level and C : P ratio. Animal's P contents measured at end of each experiment.

Table 2. Phosphorus homeostasis coefficients (H) for cladocerans fed algae differing in P content. H is the inverse of the slope of the linear regression between the log of %P of animal and the log of %P of algae in each diet. Larger H values indicate tighter elemental homeostasis.

Cladoceran	Low food	High food
Ceriodaphnia	57.7	50.3
D. ambigua	13.9	7.1
D. pulex	16.7	8.1
Moina	313.0	34.6

Table 3. Carbon, nitrogen, and phosphorus concentrations and C : N and C : P ratios (molar) of all food sources used in the second growth bioassay. Numbers in parentheses are the calculated C : P ratios after the addition of supplements to P-deficient food.

Food/supplement	C (μg L^{-1})	N (μg L^{-1})	P (μg L^{-1})	C : N	C : P
P$^-$Ank	371.8	34.9	1.4	12.4	690
+PO_4	371.8	34.9	2.7	12.4	360
P$^+$Ank	541.0	49.9	6.8	12.6	204 (260)
Syn	183.2	41.9	6.0	5.1	78 (140)

limitation at low food, in support of the energy limitation concept. However, *Moina* was the exception. Although this species grew at very low rates in the low food treatments, it displayed a strong negative dependence on C : P ratio. A reasonable explanation for *Moina*'s strong response to P at low food was that the food was still above the threshold food concentration for this species. However, this species exhibited a large decrease in sensitivity to P limitation in low compared with high food, which supports the stoichiometry theory prediction of a shift to energy limitation at threshold food conditions. Similarly, Boersma and Kreutzer (2002) showed that *Daphnia* decreased growth rate in P-limited *Scenedesmus*, but this effect disappeared when phosphorus was added to P-deficient algae, even at very low food levels (0.03 to 0.15 mg C L^{-1}). This range includes threshold food levels reported for several cladocerans species, including tropical ones (Gliwicz 1990; Hardy and Duncan 1994; 0.05 mg C L^{-1} for *Moina*; 0.08 mg C L^{-1} for *Ceriodaphnia*, A. S. Ferrão-Filho unpubl. data). Thus, the shift from elemental to energy limitation may not be so simply related to food concentration as currently incorporated in stoichiometric models.

Our results confirm the findings of others demonstrating that the assumption of strict consumer homeostasis of the stoichiometric theory needs to be relaxed. Two of our study species showed weak homeostasis (low *H*) in P content along the C : P ratio gradient, both in low and high food levels. Interestingly, *Ceriodaphnia* and *Moina*, the two smallest cladocerans, showed relatively tight homeostasis (high *H*) in P content along the C : P ratio gradient, despite being quite different in mean P content and in the sensitivity to P-deficient diet. DeMott and Pape (2005) also document striking deviations from strict homeostasis, and further show that the magnitude of P content decline in P-deficient diet varied considerably among 10 daphniid taxa. These results emphasize that animal plasticity in P content and in sensitivity of growth to P deficiency are highly variable across taxa. The two most homeostatic species (*Ceriodaphnia* and *Moina*) were the most disparate in growth rates and in sensitivity of growth to P deficiency. Thus, in agreement with DeMott and Pape (2005), our results are contrary to the hypothesis that tightness of homeostasis can be used to predict sensitivity to element limitation.

Despite the conflict with this aspect of stoichiometric theory, the tendency of animals to show tighter P homeostasis at low food levels is in agreement with the idea that, at strong P limitation, animals decrease P release in order to limit decreases in the P content of their tissues. DeMott et al. (1998) have shown, however, that even at strong P limitation, *Daphnia* continues releasing phosphorus. Anderson et al. (2005) suggested a mechanistic model in which animals regulate the excess of C, N and P by what they called "postabsorptive process," in order to reach homeostasis. They demonstrated using P-limited *Scenedes-*

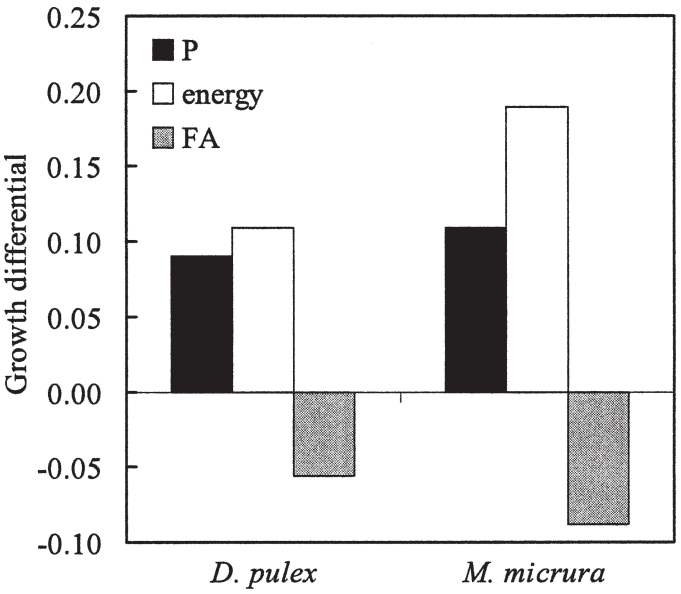

Fig. 3. Growth differentials for phosphorus (P), energy, and fatty acids (FA) in supplementation experiment. Growth differential is difference in mean growth rate between specific treatments.

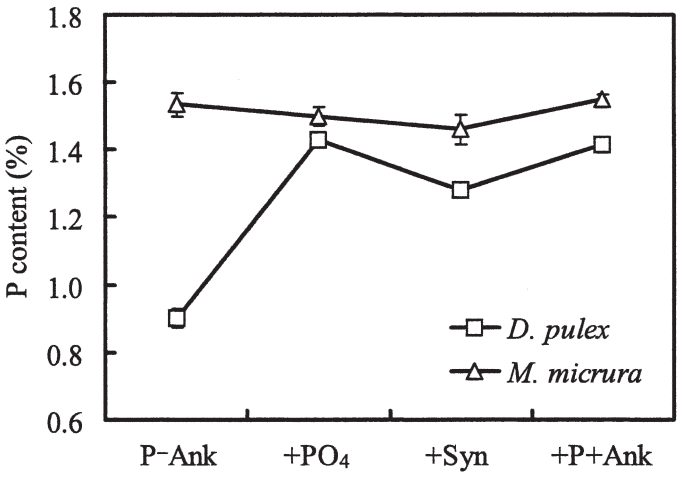

Fig. 4. Changes in P balance of *D. pulex* and *M. micrura* in supplementation experiment. Error bars are standard deviation.

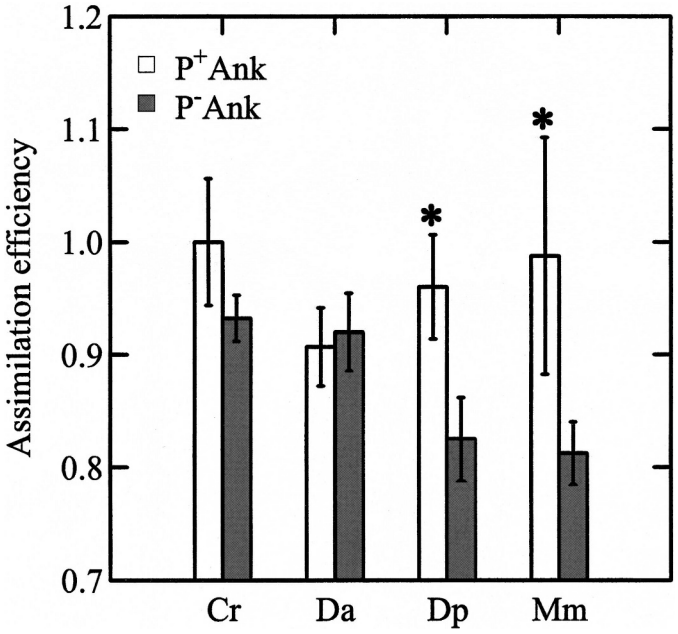

Fig. 5. Assimilation efficiencies of cladocerans feeding on P^+Ank and P^-Ank. Cr = *Ceriodaphnia*; Da = *D. ambigua*; Dp = *D. pulex*; Mm = *Moina*. Error bars are standard error. $*p < 0.05$.

mus that P excretion due to P turnover in biomass is constant and low, and fitted perfectly the data from DeMott et al. (1998). Thus, the tighter P homeostasis in *Ceriodaphnia* and *Moina* suggests an even more efficient mechanism of postabsorption of nutrients than *Daphnia*. However, studies of P excretion rate of these two species are needed to solve the paradoxical finding of how species differing highly in growth rate potential and in sensitivity to P-limited growth can have such tight P homeostasis.

Our data also provide support for the hypothesis that animals with higher P content exhibit higher maximal growth rates. The two highest P content species (*D. ambigua* and *Moina*) were the most sensitive to growth depression in P deficient food, and also exhibited the highest maximum growth rates at high food levels and with P-sufficient food. These results support the GRH (Elser et al. 2003) and the prediction of Sterner and Hessen (1994) that sensitivity to P deficiency will vary with P content of the herbivore. Only a few previous studies have examined this relationship (DeMott et al. 1998; DeMott 2003). DeMott and Pape (2005) found no consistent relationship between consumer's P content and either maximal growth rate or sensitivity to P limitation in comparisons among 10 *Daphnia* taxa. Elser et al. (2003) also showed uncoupling between P content and growth rate in *D. pulex* when animals were grown in very low food levels (0.1 mg C L^{-1}) but they did not test how variation in C : P ratios of food can influence this relationship. In support of GRH, Acharya et al. (2004*a*) found a tight correlation between growth rate, %RNA and %P under P limitation but not under N limitation. We provide a more rigorous test of GRH than these previous studies because we used a continuous gradient in the C : P ratio and showed how this relationship between growth rate and P content breaks down at low food levels and at high C : P ratios by using quite different cladoceran taxa.

Our second growth experiment revealed differences in sensitivity of *D. pulex* and *Moina* to P-deficient food. Although *D. pulex* showed moderate response to both P and energy, *Moina* showed a stronger response to energy (+Syn) addition. However, after PO_4 addition the C : P ratio was still above the C : P threshold ratio (>300; Sterner and Elser 2002), suggesting that animals could still be limited by P, and complicating the interpretation of energy limitation in +Syn, because C : P ratio in this treatment was even lower than in the $+PO_4$ treatment. On the other hand, lower assimilation efficiency in P-deficient food may have reduced carbon availability to animals. In this case, even if the PO_4 addition was enough to reduce C : P ratios below threshold levels, animals might have been also limited by energy. However, these interpretations must be viewed with caution because we are comparing treatments that differ in the nature of the resources added to P-deficient food. Although in the $+PO_4$ treatment the resource added is an inorganic source of phosphorus, in the +Syn treatment, a second source containing both P and carbon, besides other biochemicals, was added. Acharya et al. (2004*b*) showed that *Daphnia* growth rates did not differ significantly when fed algal mixtures (MIX) or uniform (UNI) algal diets, both having virtually the same moderately high C : P ratio (~460). They also showed that this was true for *D. galeata* feeding at both low (0.25 mg C L^{-1}) and high (1.5 mg C L^{-1}) food levels, but not for *D. pulicaria* at the higher food level. One possibility argued by these authors is that P-limited algae may vary in the abundance of unsaturated fatty acids and sterols (Brett and Müller-Navarra 1997; Von Elert 2002) and thus the higher growth rate of *D. pulicaria* on the MIX diet may have reflected the contribution of these biochemicals by the high phosphorus (C : P~110) cells in the mixture. In our growth experiment with supplements of P^+Ank to P^-Ank, however, we found no evidence that the lack of unsaturated fatty acids limited the growth of the animals.

Our ^{32}P isotope method may have underestimated P assimilation efficiency if the animals excreted the recently ingested radioactive P during the 48-min trials. However, our experiment should provide a conservative estimate of the effect of P-sufficient and P-deficient diets, because animals feeding on P-sufficient diets are expected to excrete a higher proportion of ingested P than animals feeding on P-deficient diets (Anderson et al. 2005). Therefore, even considering P loss during the experiments, our P assimilation measures can be considered conservative estimates of the differences in digestibility between the P-deficient and the P-sufficient green algae. However, in the absence of measures of P excretion we cannot rule out that the lower P assimilation in low P food was caused by other mechanisms than digestion resistance.

Digestion resistance has been documented as a major food constraint for zooplankton growth in lakes (DeMott and Tessier 2002; DeMott et al. 2004*b*, Ferrão-Filho and Arcifa 2006) and can be accentuated by P deficiency in algae (Van Donk and Hessen 1993; Van Donk et al. 1997). Our P^-Ank cells appeared bigger and darker, suggestive of

a thickening of the cell walls. Although overall assimilation was very high (~80–90%), two of the four cladocerans had significantly lower assimilation rates when fed P-deficient algae. Ferrão-Filho et al. (2005) suggested that *Moina* may be especially sensitive to digestion resistance in natural phytoplankton, given its high food requirements. In contrast, DeMott et al. (1998) found no evidence of digestion resistance in experiments with a P-limited green alga and *Daphnia magna*. Thus, variability in species response to digestibility of resources suggests yet another aspect to the issue of adaptation to food quality in nature.

Our results emphasize that stoichiometric theory is too simplistic to fully explain patterns of mass balance in cladocerans of different size and life histories. An alternative framework is the dynamic energy budget (DEB) model, a more complex approach that describes the rates at which an organism acquires and utilizes energy [and elements] from its environment (Kooijman 1995). An important aspect of DEB theory is that organisms are built out of permanent (structure) and nonpermanent (reserves) components, which are considered separated pools. The reserves are much more dynamic, require no maintenance, and may differ in composition compared with the structure of the animal (Kooijman 2001). Consequently, DEB allows for weak homeostasis of elements because structure and reserves density may change relative to each other such that whole body composition changes. The extent to which organisms maintain homeostasis may reflect different strategies of species to deal with changing environmental conditions, e.g. how they manage their internal reserves. For example, *Moina*, a small fast-growing species, may allocate much energy to growth at the juvenile stage, being very sensitive to energy limitation (Ferrão-Filho et al. 2005; Ferrão-Filho and Arcifa 2006). This cladoceran fits well the ''bang-bang'' allocation strategy (Lika and Kooijman 2003) in which growth of a determinate organism ceases at maturity and all energy flowing out of the reserves is allocated to maintenance and reproduction.

In conclusion, our results suggest that the assumption of strict element homeostasis needs to be relaxed to account for variation among species. However, we did find good support for the GRH and its adaptive relevance to high food quantity and quality environments. Also, the finding that magnitude of homeostasis in body P contents (H value) is not coupled with growth sensitivity to low P content food suggests that different species have different physiological strategies to cope with changing food environments. Finally, our findings of lower P assimilation efficiency for some species when feeding on P-deficient algae suggests that differences in food digestibility (i.e., food density in the DEB model) and assimilation efficiency of consumers should be incorporated in future stoichiometric models.

References

Acharya, K., M. Kyle, and J. J. Elser. 2004*a*. Effects of stoichiometric dietary mixing on *Daphnia* growth and reproduction. Oecologia **138:** 333–340.

———, ———, and ———. 2004*b*. Biological stoichiometry of *Daphnia* growth: An ecophysiological test of the growth rate hypothesis. Limnol. Oceanogr. **49:** 656–665.

Anderson, T. R., and D. O. Hessen. 1995. Carbon or nitrogen limitation in marine copepods? J. Plankton Res. **17:** 317–331.

———, ———, J. J. Elser, and J. Urabe. 2005. Metabolic stoichiometry and the fate of excess of carbon and nutrients in consumers. Am. Nat. **165:** 1–15.

Boersma, M., and C. Kreutzer. 2002. Life at the edge: Is food quality really of minor importance at low quantities? Ecology **83:** 2552–2561.

Brett, M. T., and D. C. Müller-Navarra. 1997. The role of highly unsaturated fatty acids in aquatic food web processes. Freshwat. Biol. **38:** 483–499.

———, ———, and S. K. Park. 2000. Empirical analysis of the effect of phosphorus limitation on algal food quality for freshwater zooplankton. Limnol. Oceanogr. **45:** 1564–1575.

Darchambeau, F., P. J. Færøvig, and D. O. Hessen. 2003. How *Daphnia* copes with excess carbon in its food. Oecologia **136:** 336–346.

Demott, W. R. 1998. Utilization of a cyanobacterium and a phosphorus-deficient green alga as complementary resources by daphnids. Ecology **79:** 2463–2481.

———. 2003. Implications of element deficits for zooplankton growth. Hydrobiologia **491:** 177–184.

———, and B. J. Pape. 2005. Stoichiometry in an ecological context: Testing for links between *Daphnia* P-content, growth rate and habitat preference. Oecologia **142:** 20–27.

———, and A. J. Tessier. 2002. Stoichiometry constraints vs. algal defenses: Testing mechanisms of zooplankton food limitation. Ecology **83:** 3426–3433.

———, J. R. Edington, and A. J. Tessier. 2004*a*. Testing zooplankton food limitation across gradients of depth and productivity in small stratified lakes. Liminol. Oceanogr. **49:** 1408–1416.

———, R. D. Gulati, and E. V. Donk. 2001. Effects of dietary phosphorus deficiency on the abundance, phosphorus balance, and growth of *Daphnia cucullata* in three hypereutrophic Dutch lakes. Limnol. Oceanogr. **46:** 1871–1880.

———, ———, and K. Siewertsen. 1998. Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. Limnol. Oceanogr. **43:** 1147–1161.

———, B. J. Pape, and A. J. Tessier. 2004*b*. Patterns and sources of variation in *Daphnia* P-content in nature. Aquat. Ecol. **38:** 433–440.

Elser, J. J. 2000. Ecological stoichiometry: From sea to lake to land. TREE **15:** 393–394.

———, K. Acharya, M. Kyle, J. Cotner, W. Makino, T. Markow, T. Watts, S. Hobbie, W. Fagan, J. Schade, J. Hood, and R. W. Sterner. 2003. Growth rate–stoichiometry couplings in diverse biota. Ecol. Lett. **6:** 936–946.

———, W. F. Fagan, R. F. Denno, D. R. Dobberfuhl, A. Folarin, A. Huberty, S. Interlandi, S. S. Kilham, E. McCauley, K. L. Schulz, E. H. Siemann, and R. W. Sterner. 2000*a*. Nutritional constraints in terrestrial and freshwater food webs. Nature **408:** 578–580.

———, R. W. Sterner, E. Gorokhova, W. F. Fagan, T. A. Markow, J. B. Cotner, F. Harrison, S. E. Hobbie, G. M. Odell, and L. J. Weider. 2000*b*. Biological stoichiometry from genes to ecosystems. Ecol. Lett. **3:** 540–550.

Ferrão-Filho, A. S., and M. S. Arcifa. 2006. Growth responses of tropical cladocerans to seston from Lake Monte Alegre (Brazil) supplemented with phosphorus, fatty acids, a green algae and a cyanobacterium Hydrobiologia **563:** 155–165.

———, W. R. Demott, and A. J. Tessier. 2005. Response of tropical cladocerans to a natural gradient of resource quality. Freshw. Biol. **50:** 954–964.

Gliwicz, Z. M. 1990. Food thresholds and body size in cladocerans. Nature **343:** 638–640.

Hardy, E. R., and A. Duncan. 1994. Food concentration and temperature effects on life-cycle characteristics of tropical Cladocera *Daphnia gessneri* Herbst, *Diaphanosoma sarsi* Richard, and *Moina reticulata* Daday. I. Development time. Act. Amaz. **24:** 119–134.

Kooijman, S. A. L. M. 1995. The stoichiometry of animal energetics. J. Theor. Biol. **177:** 139–149.

———. 2001. Quantitative aspects of metabolic organization: A discussion of concepts. Phil. Trans. R. Soc. Lond. B **356:** 331–349.

Lika, K., and S. A. L. M. Kooijman. 2003. Life history implications of allocation to growth versus reproduction in dynamic energy budgets. Bull. Math. Biol. **65:** 809–834.

Plath, K., and M. Boersma. 2001. Mineral limitation of zooplankton: Stoichiometric constraints and optimal foraging. Ecology **82:** 1260–1269.

Sterner, R. W. 1997. Modelling interactions of food quality and quantity in homeostatic consumers. Freshw. Biol. **38:** 473–481.

———. 2000. Nutritional constraints in terrestrial and freshwater food webs. Nature **408:** 578–580.

———, and J. J. Elser. 2002. Ecological stoichiometry: The biology of elements from molecules to the biosphere. Princeton Univ. Press.

———, and D. O. Hessen. 1994. Algal nutrient limitation and the nutrition of aquatic herbivores. Ann. R. Ecol. **25:** 1–29.

———, and J. L. Robinson. 1994. Threshold for growth in *Daphnia magna* with high and low phosphorus diets. Limnol. Oceanogr. **39:** 1228–1232.

Tessier, A. J., and P. Woodruff. 2002. Trading off the ability to exploit rich versus poor food quality. Ecol. Lett. **5:** 685–692.

Tollrian, R. 1993. Neckteeth formation in *Daphnia pulex* as an example of continuous phenotypic plasticity: Morphological effects of *Chaoborus* kairomone concentration and their quantification. J. Plankton Res. **15:** 1309–1318.

Van Donk, E., and D. O. Hessen. 1993. Grazing resistance in nutrient stressed phytoplankton. Oecologia **93:** 508–511.

———, M. Lürling, D. O. Hessen, and G. M. Lokhorst. 1997. Altered cell morphology in nutrient-deficient phytoplankton and its impact on grazers. Limnol. Oceanogr. **42:** 357–364.

Von Elert, E. 2002. Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. Limnol. Oceanogr. **47:** 1764–1773.

Received: 29 July 2005
Amended: 6 July 2006
Accepted: 2 August 2006