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The role of photosynthetic capacity and of leaf area ratio for the carbon gain of C3 and C4 species during the cool season in the Río de la Plata grasslands

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ABSTRACT

The proportion of C4 species as a component of the aboveground biomass of grasslands becomes typically less with decreasing temperatures, in either latitudinal, altitudinal or seasonal gradients. This correlation is believed to be caused by the differential responses of C3- and C4-photosynthesis to temperature. However, such a mechanism has not been explicitly tested in field stands. The present research addressed the photosynthetic performance during the cool-season of individuals of several dominant C3 and C4 species growing in subtropical and temperate grasslands of Argentina and Uruguay (Río de la Plata grasslands). Specifically, this study analyzed the role of physiological (carbon gain per unit leaf area) and morphological (leaf area per shoot) determinants of the daily carbon gain of C3 and C4 individuals growing in natural field stands during the autumn-to-winter transition.

The first part of this thesis describes and assesses a new approach to measure *in situ* daily gross carbon gain of individuals growing in mixed canopies, based on measuring the ^{13}C-content of shoots after a few hours of continuous ^{13}C-labeling of assimilated CO_2. Results from two labeling experiments (Exps. 1 and 2) carried out in grasslands of Germany and Argentina, and from a survey of literature data, showed that this technique can accurately quantify carbon gain, underestimating it by less than 10% if the duration of the labeling period is kept below six or seven hours (so that tracer losses from shoots, *via* either respiration or belowground partitioning, are minimal).

In the second part of the thesis, five labeling experiments (Exps. 3 to 7), also performed in grasslands of Germany and Argentina, demonstrated that carbon gain per unit shoot mass can have an asymptotic relationship with individual's size, and that this is related to the hierarchical position (subordinate or dominant) of the individuals within the stand. Thus, irrespectively of mass or growth form (*e.g.* rosette dicots *vs.* grasses), being taller than neighbors is most important in determining the carbon gain per unit shoot mass of individuals. In consequence, differences between functional groups in their dominance, *i.e.* contribution to standing biomass, are not necessarily a good indicator of which functional group is actually gaining, that is, is increasing its contribution.

In the third part of this thesis, a set of seven additional labeling experiments (Exps. 8 to 14) performed at a temperate and a subtropical site of the Río de la Plata

grasslands served to assess the role of carbon fixation per unit leaf area and leaf area per unit shoot mass on the performance of C3 and C4 individuals during the cool-season. To avoid the effects of individual's size and hierarchy on carbon gain, a constant sward surface height close to ~ 0.10 m was kept throughout the year by a regime of continuous grazing with variable stocking rates. Carbon gain per unit shoot mass decreased relatively more in C4 than in C3 plants during the autumn-to-winter transition, in both subtropical and temperate climates. In the former, this was largely due to a reduced photosynthetic capacity of C4 leaves. Conversely, at the temperate site, the lesser performance of C4 species was entirely due to a morphological constraint, as the amount of leaf area per unit mass of C4 individuals decreased drastically in winter.

These results demonstrate (i) the general capability of the new labeling approach to test carbon gain-based hypotheses, in natural stands, under field conditions; (ii) a prominent role for leaf photosynthesis in determining the C3/C4 balance over the cool-season in subtropical grasslands; and (iii) a prominent role of morphological processes on the poor performance of C4 species during the cool-season in temperate grasslands. While C4 vegetation dominates subtropical grasslands, the cool-season of temperate areas defines the boundary for the distribution of C4 ecosystems. The results of the present thesis therefore suggest that the expansion of C4 species along latitudinal gradients may, in fact, be limited by the ability to develop and sustain leaf area, rather than by the photosynthetic performance of leaves.

ZUSAMMENFASSUNG

Mit sinkender Umgebungstemperatur sinkt der Bestandesanteil der C4-Pflanzen im oberirdischen Aufwuchs von Grünlandbeständen. Dieser Sachverhalt gilt sowohl für die jahreszeitlichen Änderungen in der Umgebungstemperatur, wie auch für Änderungen welche sich entlang von Längen- und Breitengraden ergeben. Die Ursache dieses Zusammenhangs wird der unterschiedlichen Temperaturabhängigkeit der C3- und C4-Photosynthese zugeschrieben. Allerdings wurde dieser Mechanismus bisher noch nicht explizit im Feld untersucht. Die vorliegende Forschungsarbeit beschäftigte sich mit der Photosyntheseleistung von Pflanzen mehrerer dominanter C3- und C4-Arten des subtropischen und gemäßigten Grünlands Argentiniens und Uruguays (Río de la Plata-Grünland) während der kühlen Jahreszeit. Dabei wurde insbesondere die Rolle physiologischer (Kohlenstoffgewinn je Einheit Blattfläche) und morphologischer (Blattfläche je Trieb) Bestimmungsgrößen des täglichen Kohlenstoffgewinns von in natürlichen Beständen wachsenden C3- und C4-Einzelpflanzen während des Übergangs von Herbst zu Winter untersucht.

Der erste Teil dieser Arbeit beschreibt und beurteilt eine neue Methode zur in-situ-Messung des täglichen Kohlenstoffgewinns von in gemischten Beständen wachsenden Individuen, die auf einer Messung des ^{13}C-Gehalts der Triebe nach einigen Stunden kontinuierlicher Markierung des assimilierten CO_2 beruht. Zwei in Deutschland und Argentinien durchgeführte Markierungsexperimente (Exp. 1 und 2) und eine Recherche der Literaturdaten zeigten, dass diese Technik den Kohlenstoffgewinn akkurat bestimmt. Bei Markierungsperioden von maximal sechs oder sieben Stunden (wodurch die durch Atmung oder durch Verteilung im Boden hervorgerufenen Tracerverluste im Spross minimal gehalten werden) wird der Kohlenstoffgewinn um weniger als 10% unterschätzt.

Im zweiten Teil der Doktorarbeit konnte durch fünf Markierungsexperimente in Gründlandbeständen Deutschlands und Argentiniens (Exp. 3 bis 7) nachgewiesen werden, dass der Kohlenstoffgewinn je Einheit Sprossmasse ein asymptotisches Verhältnis zur Größe der Einzelpflanzen haben kann und dass dies mit der hierarchischen Stellung (untergeordnet oder dominant) der Individuen im Bestand zusammenhängt. Unabhängig von Masse oder Wuchsform (z.B. zweikeimblättrige Rosettenpflanzen im Vergleich zu Gräsern) ist daher bei Einzelpflanzen der Höhenvorteil im Vergleich zur Nachbarpflanze der wichtigste Faktor für den

Kohlenstoffgewinn je Einheit Sprossmasse. Folglich sind Dominanzunterschiede zwischen funktionellen Gruppen, z.B. ihre Anteile an der stehenden Biomasse, nicht notwendigerweise ein guter Indikator dafür, welche funktionelle Gruppe überwiegt beziehungsweise ihren Anteil erhöht.

Im dritten Teil dieser Arbeit diente eine Reihe von sieben weiteren, an gemäßigten und subtropischen Versuchsstandorten des Río de la Plata-Grünlands durchgeführten Markierungsexperimenten (Exp. 8 bis 14) dazu, die Rolle der Kohlenstofffixierung je Einheit Blattfläche und der Blattfläche je Einheit Sprossmasse auf die Photosynthese von C3- und C4-Pflanzen während der kühlen Jahreszeit zu bewerten. Um den Einfluß der Hierarchie und der Wuchshöhe der Individuen auf den Kohlenstoffgewinn zu unterbinden, wurden die Bestände durch kontinuierliche Beweidung mit variabler Besatzdichte das ganze Jahr über auf konstanter Höhe von ~0.10 m gehalten. Sowohl im subtropischen als auch im gemäßigten Klima ging während des Herbst-Winter-Übergangs der Kohlenstoffgewinn je Einheit Sprossmasse bei den C4-Pflanzen relativ stärker zurück als bei den C3-Pflanzen. An den subtropischen Versuchsstandorten beruhte dies hauptsächlich auf einen Rückgang der Photosyntheseleistung der C4-Blätter. Umgekehrt war die geringere Leistung der C4-Arten am gemäßigten Versuchsstandort vollständig auf einen morphologisch begrenzenden Faktor zurückzuführen, da die Blattflächenmenge je Einzelpflanze im Winter drastisch zurückging.

Diese Ergebnisse belegen (1) das Potential der neuen Markierungstechnik zur Prüfung von Hypothesen bezüglich des Kohlenstoffgewinns in natürlichen Beständen unter Feldbedingungen; (2) eine herausragende Rolle der Blatt-Photosynthese auf das C3/C4-Verhältnis während der kühlen Jahreszeit im subtropischen Grünland und (3) eine große Bedeutung morphologischer Prozesse für die geringe Photosyntheseleistung der C4-Arten gemäßigter Zonen. Während die C4-Arten im subtropischen Grünland überwiegen, stellt die kühle Jahreszeit der gemäßigten Zonen den die Ausbreitung der C4-Ökosysteme begrenzenden Faktor dar. Die Ergebnisse der vorliegenden Doktorarbeit legen daher nahe, dass die Breitengrad-Ausdehnung von C4-Arten eher durch die Fähigkeit, Blattfläche zu entwickeln und zu erhalten, als durch die Photosyntheseleistung der Blätter begrenzt ist.

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I. GENERAL INTRODUCTION

It is well known that in mixed C3/C4 grasslands the proportion of C4 species as a component of the aboveground biomass decreases as latitude and/or altitude increase. Similarly, in these ecosystems the contribution of C4 biomass typically decreases as the cool-season progresses from end of summer, to autumn, to winter. These patterns have been found to correlate well with growing season mean temperature, and with summer temperatures in particular (Terri and Stowe 1976; Hatterseley 1983; Tieszen et al. 1997, Cabido et al. 1997). It is thus generally thought that at low temperatures the performance of C4 plants is intrinsically less than that of C3 species (see reviews by Long 1999; Sage et al. 1999; Sage and Pearcy 2000 and references therein).

Ecophysiological analyses of C3/C4 interactions have been centered on the photosynthetic response of C3 and C4 species to temperature (Sage and Pearcy 2000). The rationale behind this emphasis is that photosynthesis is the ultimate source of substrates for growth and reproduction (Anten 2005), and that the photosynthetic metabolism is remarkably different between C3 and C4 species (Hatch et al. 1975; Hatch 1992). The fact that the discovery of the C4 pathway coincided with the development of the infrared gas analyzer –a technology allowing accurate and rapid measurements of instantaneous photosynthetic rates– may have also played a role in explaining the dominance of photosynthesis-based analyses. It certainly lead scientists to re-examine the role of photosynthesis in plant growth (*e.g.* Duncan and Hesketh 1968; Potter and Jones 1977; Baskin and Baskin 1978 and references therein; Poorter et al. 1990).

The essential difference between the C3 and C4 modes of photosynthesis is that in the latter, the CO_2 partial pressure (pCO_2) at the site of Rubisco in bundle-sheath cells is elevated more than 10 times by a biochemical CO_2-pumping

mechanism. This elevated pCO_2 effectively prevents photorespiration by suppressing O_2 competition at Rubisco. Since photorespiration is strongly enhanced by temperature, net photosynthetic rates are higher in C4 than C3 plants at high temperatures (Pearcy et al. 1981; Kubien and Sage 2004a,b; Sage and Pearcy 2000). Further, such a CO_2 concentrating mechanism allows Rubisco to operate at (nearly) CO_2-saturated rates in C4 leaves. Therefore, photosynthesis typically saturates at a higher light intensity in C4 than in C3 plants (Long 1999), specially as temperatures increase (Pearcy et al. 1981; Kubien and Sage 2004a).

The CO_2-pump involves assimilation of CO_2 into a four-carbon organic acid (hence the name) by Phosphoenolpyruvate carboxylase (PEP) in mesophyll cells, followed by its transport and subsequent decarboxylation in bundle sheath cells. The regeneration of pyruvate in the mesophyll requires the use of 2 ATP for each pumped CO_2 molecule, which are produced by photophosphorylation at a rate of ~ 8 photons per ATP (Furbank et al. 1990). Pumping costs are further increased by the fact that some of the CO_2 leaks back to the mesophyll (Farquhar 1983). Therefore, the maximum quantum yield –that is, the moles of CO_2 fixed per mol of absorbed photons at very low light intensity (termed 'quantum yield', hereafter)– is lower in C4 than in C3 plants. However, at high temperature (or low intercellular pCO_2), photorespiratory costs more than offset the ATP consumption of the C4 pump, and quantum yield becomes higher in C4 than in C3 plants (Ehleringer and Bjorkman 1977; Ehleringer and Pearcy 1983).

Ehleringer (1978) formally introduced the above-described interaction between photosynthetic pathway and temperature into a canopy photosynthesis model and reported that spatial and seasonal patterns of C3 *vs.* C4 abundance agreed well with the predicted C3/C4 balance of carbon gain rates. This model, later simplified to

a single equation relating quantum yield of C3 and C4 plants to temperature and atmospheric pCO_2, has been used to assess the evolution of C4 taxa (*e.g.* Ehleringer et al. 1997) and the worldwide distribution of C3/C4 ecosystems under present, past and future atmospheric conditions (*e.g.* Collatz et al 1998).

The validity of this model, hereafter referred to as the quantum yield hypothesis, has been criticized on several grounds (*e.g.* Winslow et al. 2003; Osborne and Freckleton 2009; Edwards et al. 2010). However, to my knowledge, it is the only hypothesis on the balance between C3 and C4 species able to provide an explicit, testable prediction regarding the C3/C4 balance of grasslands. It predicts that at current atmospheric pCO_2, C3 plants should take advantage at daytime temperatures below 21–26 ºC because their leaf-level carbon gain is higher than that of C4 plants. As far as I know, this has not been tested in natural vegetation, growing under field conditions.

The main aim of this thesis was to analyze the carbon gain of individuals of the C3 and C4 functional groups growing in mixed natural grasslands, during the autumn-to-winter transition, in both subtropical and temperate areas. For this, a new approach to quantify carbon gain was developed based on ^{13}C-labeling. Daily carbon gain per unit shoot mass was analyzed following Poorter (1989) in terms of the relative importance of a 'physiological component', the carbon gain per unit leaf area, and a 'morphological component', the amount of leaf area per unit shoot carbon mass. Three specific questions were asked: (*i*) is the well documented decrease in abundance of C4 species during the autumn-to-winter transition associated with an impaired ability to gain carbon? If so, (*ii*) is such a reduction of daily carbon gain in C4 plants due to decreases in leaf area or in photosynthetic capacity? Finally, (*iii*) are differences in carbon gain between C3 and C4 individuals linked to differences in

photosynthetic capacity or to differences in the amount of leaf area to fix carbon?

In the present thesis, all materials and methods are presented in Chapter II (p. 11). The presentation of results (Chapter III, p.27) and its discussion (Chapter IV, p.42) are both structured around three, interrelated parts:

(a) The first part describes a novel approach to measure the daily carbon gain of individuals growing in dense field canopies based on the continuous ^{13}C-labeling of all assimilated CO_2 at a constant enrichment level, followed by the immediate harvest of labeled shoots and determination of their ^{13}C content. Results from test trials on which tracer content during labeling was quantified in above- and belowground organic carbon pools (Exps. 1 and 2), and from a survey of data on the literature, both served to assess the method.

(b) The second part analyzes the relationship between the daily carbon gain and the size and hierarchical position of individuals and functional groups, growing in either short and tall, sunlit or shaded, field stands (Exps. 3 to 7).

(c) The third part addresses the main aim of the thesis, and thus answers the questions posed above. It presents the results from a set of seven additional labeling experiments (Exps. 8 to 14) performed in two seasons (autumn and winter) and at two sites (a temperate and a subtropical one) located in the Río de la Plata grasslands (Argentina and Uruguay).

The essence of parts (a) and (b) has been published recently in Ecology (Lattanzi, Berone, et al. *in press*).

In what follows, the methodologies available for measuring the carbon gain of individuals under field conditions are briefly introduced and the advantages that the

new labeling method brings about, described. Further, the effects that individual's size and hierarchical position can have upon carbon gain are exemplified with the interaction between rosette dicots and grasses in short *vs.* tall canopies. Then, the performance of C4 *vs.* C3 species in grasslands in cool environments is concisely reviewed, with emphasis on aspects that remain poorly understood, and the scope of the measurements carried out in this work is briefly described.

I.1 Assessing the carbon gain of individuals in multi-specific field stands

Mechanistic analyses of plant competition, and thus our understanding of species coexistence, would improve if the amount of carbon assimilated over the course of a day by individuals growing in field stands were easy to measure. For instance, the 'which species/functional group is gaining'-question –central to the present thesis– could be addressed by assessing the daily carbon gain per unit biomass of all species or functional groups present in a stand (Connolly et al. 2001). Further, and also most relevant to this thesis, such a measurement would serve to identify which traits affect the most the carbon gain of individuals competing in multispecies or hierarchically structured stands (*e.g.* Gaudet and Keddy 1988; Ramseier and Weiner 2006).

Daily carbon gain of individuals has usually been estimated by models that scale-up leaf photosynthesis (*e.g.* Barnes et al. 1990; Hikosaka et al. 1999; Anten and Hirose 2003). This approach has the advantage of explicitly accounting for all components influencing carbon gain of individual leaves. But it requires determining a relatively large number of parameters: it involves measuring the response of leaf photosynthesis to irradiance and nitrogen, and then integrating it across all leaves of an individual, which in turn requires measurements of both the diurnal course of photosynthetic active radiation (PAR) at several canopy depths and the distribution

and geometry of leaves within the canopy. For this reason, it becomes difficult to simultaneously measure many species, or a whole functional group. Further, several assumptions are made. Two of the most important ones are that photosynthetic and respiratory responses to PAR and nitrogen of each species are equal for all leaves, and that effects of diurnal and spatial (within canopy) variations in other factors (*e.g.* leaf temperature, leaf age) are negligible.

In this thesis, an alternative method to estimate carbon gain is presented. It is based on the continuous ^{13}C-labeling of all assimilated CO_2 at a constant enrichment level (*i.e.* steady state labeling), followed by the immediate harvest of labeled plants and determination of their ^{13}C content. In comparison, this approach does not provide information on the carbon gain of different parts of an individual: *e.g.* leaves at different positions within the canopy. On the other hand, it assumes that tracer losses from shoots over the labeling period should be low (which is assessed). Most importantly, estimating the carbon gain of all species or functional groups in a stand becomes as simple as harvesting all present species or functional groups.

Steady state labeling is a well established technique, with set ups designed for growth chambers (Deléens et al. 1983; Geiger and Shieh 1988; Schnyder 1992), mesocosms (Klumpp et al. 2007), and open top chambers in the field (Gamnitzer et al. 2009). A labeling approach has been previously used to measure daily carbon gain of sand-potted plants growing in controlled environments (Lattanzi et al. 2005; Grimoldi et al. 2006). Here a fully mobile facility for steady state labeling of natural vegetation with minimal disturbance of the stand is presented. Such portability is unique for a labeling set up, and greatly extends its actual usability (Liu et al. 2000).

The performance of the labeling facility was tested on several preliminary trials, and the assumptions of the method –in particular, that tracer losses from shoots

over the labeling period are relatively low– assessed from experimental results and from a survey of literature data (sections III.1 and IV.1). Once the applicability and accuracy of the method were established, the potential of the approach to test carbon gain-based hypotheses in natural stands under field conditions was demonstrated. Specifically, the effects of size and hierarchy of individuals on their carbon gain were explored (sections III.2 and IV.2).

For this, experiments carried out in grasslands of Argentina and Germany served to, firstly, analyze the relationship between size and carbon gain for individuals of coexisting species growing in contrasting hierarchical positions. For instance, positive relationships between individual's size and carbon gain per unit shoot mass have been reported in crowded stands (Anten and Hirose 2003). But this seems not to be always the case (Hikosaka et al. 1999). Reasons why such a relationship may become asymptotic were discussed. Secondly, the carbon gain of contrasting functional groups was estimated, analyzing the interaction between rosette dicots and grasses when growing in short *vs.* tall canopies, or in sunlit *vs.* shaded conditions, to establish whether carbon gain was related to the relative contribution of functional groups to standing biomass or rather to their hierarchical position within the stand.

I.2 The performance of C4 species in cool environments

Implicit in the quantum yield hypothesis is that daily carbon gain, in particular at low irradiances, is a major determinant of the C3/C4 balance in mixed canopies. More recent appraisals of this hypothesis have indicated that its value and predictive power resides in that it depicts the general response of (the inhibition of) photorespiration in C3 plants to (low) temperature and (high) pCO_2, and therefore it would be also valid

for light-saturated photosynthesis (Still et al. 2003; Ehleringer 2005; Kubien and Sage 2004a). This is important because in most grasslands growth is typically either nutrient- or water-limited, and grazing is ubiquitous. As a result, grassland canopies rarely sustain a high leaf area index (LAI) for any extended period of time (Knight 1973; Sala et al. 1986), and leaves are often exposed to relatively high light intensities. Thus, C3 *vs.* C4 differences in carbon gain should be explained by differences in the temperature-response of photosynthesis over the whole range of light intensities, rather than solely by differences in the temperature response of quantum yield (*i.e.* light-limited photosynthesis).

Interestingly, comparative studies have not shown a consistent pattern for C3 *vs.* C4 light-saturated photosynthesis at temperatures between 10 – 20 ºC, *i.e.* the range observe during the cool-season of many grasslands. While some studies did observe higher light-saturated photosynthesis in C3 than in C4 species (*e.g.* Kemp and Williams 1980; Pearcy et al. 1981; Fladung and Hesselbach 1987; Sage and Pearcy 2000), many other have shown equal or even higher light-saturated photosynthesis in C4 species (Cooper and Tainton 1968; Long et al. 1975; Long and Woolhouse 1978; Monson et al. 1983; Labate et al. 1990; Oberbauer and Edwards 1993; Sage 2002; Kubien and Sage 2004b; Liu and Osborne 2008; Osborne et al. 2008).

Opposite to the instantaneous assimilation rate of the youngest mature leaf, which is the typical subject of gas exchange studies, the daily carbon gain of an individual results not only from the photosynthetic capacity of its leaves but also from the amount of leaf area it sustains. Several studies have shown that, as temperatures decrease from ~30 °C to ~ 12 °C, the daily carbon gain of C4 canopies was more affected by reductions in leaf area production than by reductions in photosynthesis per unit leaf area (*Zea mays,* Duncan and Hesketh 1968; *Paspalum dilatatum,* Forde et al.

1975; *Cyperus longus,* Jones et al. 1981). Likewise, a study comparing *Spartina anglica* (a C4 grass) and *Lolium perenne* (a C3 grass) under controlled conditions demonstrated that the poorer performance of C4 individuals was mainly a consequence of their lower amount of leaf area (Dunn et al. 1987). Further, a recent analysis revealed that the higher yield of field stands of a perennial C4 grass, *Miscanthus x giganteus,* over maize crops is due to a greater ability to expand and sustain leaf area at cool temperatures early and later in the growing season, and not to a greater leaf photosynthetic capacity (Dohleman and Long 2009).

Two important points become clear: on one hand, the ecophysiological mechanisms underpinning the poor performance of C4 plants during the cool-season are still unsolved (Long 1983; Long 1999; Sage and McKown 2006). On the other hand, the role of the 'morphological component' of the daily carbon gain of individuals should be considered when C4 performance in cool environments is analyzed.

Chapter III.3 reports measurements of daily carbon gain and their components in a total of eleven C3 and C4 grasses, plus a C3 rosette dicot, growing during autumn and winter in either subtropical or temperate grasslands of Argentina and Uruguay. Seven labeling experiments were performed under field conditions. The daily carbon gain per unit shoot carbon mass (referred to as the relative gross photosynthesis rate, RPR, d^{-1}) was analyzed as the product of carbon fixed per unit leaf area (gross assimilation rate, GAR, g m^{-2} d^{-1}) and the leaf area per unit of shoot carbon mass (leaf area ratio, LAR, m^2 g^{-1}).

The RPR of C3 and C4 plants was studied as the cool-season progressed from autumn to winter in a temperate and a subtropical site of the Río de la Plata grasslands. This is a near ideal ecosystem for such an analysis since its humid climate

prevents extreme changes in soil moisture, and the absence of a 'dormant season' (no snow, absence of mean daily temperatures below zero) allows the yearlong coexistence of C3 and C4 plants with healthy, green leaves. Further, the southern (cooler) areas of these grasslands present daily mean winter temperatures of 8 °C (maximum: 13 °C, minimum: 3 °C), which are considered to limit the expansion of C4 species (Sage and Pearcy 2000).

II. MATERIALS AND METHODS

II.1 Components of the labeling facility

The labeling apparatus generated CO_2-free air, mixed it with pure CO_2 of known isotopic composition, and distributed it to transparent chambers enclosing the stands to be labeled (Figs. II.1 and II.2). The elements of the air generating unit were two: a compressor (Mobil 45G, Kaeser, Coburg, Germany) and an adsorption dryer (KEN-MT 1400 MS/TE, Zander, Essen, Germany). The compressor generated a flow of air at constant pressure, and the dryer contained a molecular sieve (Silicagel WS plus Aluminiumgel F200) that trapped CO_2 and H_2O vapor (residual $pCO_2 < 5$ μbar).

The dryer comprised two columns, each with 79 kg of molecular sieve. While one column was in operation at 7 atm, one-third of the CO_2-/H_2O-free air produced was diverted to pass through the second column (at 1 atm), to vent desorbed CO_2 and H_2O. A valve switched the air flow between columns every 5 min. The pressure and overflow requirements of this cold self-regeneration system reduced usable air flow to about 45% of the compressor technical specification, *i.e.* from 4000 L min^{-1} (at 8 atm) to 1700 L min^{-1}.

Air was filtered before (EC 48 and EE 48, Kaeser; G14XPD, Zander) and after the dryer (G14VHD, Zander). Thus cleaned from oil and dust, CO_2-/H_2O-free air was routed to a buffer tank (500 L), and then to an additional set of filters (FFG 48, Kaeser). Air flow rate and CO_2 partial pressure (pCO_2) were manually controlled *via* two digital mass flow controllers (Fig. II.1), one for CO_2-/H_2O-free air (D-6280-HAB-CC-AV-36-O-S-A, range: 150 to 3000 L min^{-1}) and one for pure CO_2 (D-5111-FAB-33-AE-99-A-S-A, range: 0.005 to 1.0 L min^{-1}. Both from MKS Instruments, München, Germany). ^{13}C-enriched CO_2 [$\delta^{13}C$ = 401.8‰, with $\delta^{13}C = (^{13}C/^{12}C_{sample})/(^{13}C/^{12}C_{PDB\ standard}) - 1$] was kept in an aluminum cylinder fitted with a

pressure regulator and Teflon tubing (Spectra Gases, Babenhausen, Germany). Mixing of the labeling CO_2 and the CO_2-/H_2O-free air occurred in a simple 'T' connector.

Air was partitioned between four groups of labeling chambers by an *air distribution unit* consisting of a set of connectors that routed the air flow to four optical volumetric flow meters, each fitted with a needle valve (VA40V/ST, range: 40 to 500 L min^{-1}, Krohne, Duisburg, Germany). Chambers located at different distances from the air mixing unit experienced different pressure drops (depending on tubing length and diameter), which translates in differences in flow detected by the flow meters. These differences were neutralized by manual adjustment of the needle valves.

Polyethylene tubing (Ø 28 mm) carried the air to the labeling chambers. To prevent heating up of circulating air, all tubes were buried 100 mm in the soil or wrapped in thermal insulation. Labeling chambers were made of acrylic glass (Plexiglas XT, 4 mm thick, Röhm Degussa, Darmstadt, Germany) held together by transparent polycarbonate profiles that could be easily disassembled for transport. Chambers had a volume of 45 L and enclosed an area of 0.16 m^2 (L · W · H: 0.57 m · 0.28 m · 0.28 m). Chambers were neither gas tight nor placed on top of any special base, but lay directly on the soil surface. The system operated with up to 20 chambers, each receiving up to 90 L min^{-1}. This implied a turnover rate of chamber air of 2.0 min^{-1} (90 L min^{-1}/45 L) corresponding to a mean residence time of air inside the chambers of half a minute (1.0/2.0 min^{-1}).

The whole facility was mobile: the air generating unit and the buffer tank were mounted on a custom made trailer (Herrería y Tornería Izarriaga, Balcarce, Argentina) that, weighting less than 3000 kg, could be towed by a common truck. All pipelines

were rolled and mounted in the trailer. The air mixing and air distribution units were mounted on a frame (L · W · H: 1.0 m · 0.6 m · 1.2 m) that fitted in the truck box, along with the acrylic panels of the (disassembled) labeling chambers and the cylinder with pure CO_2.

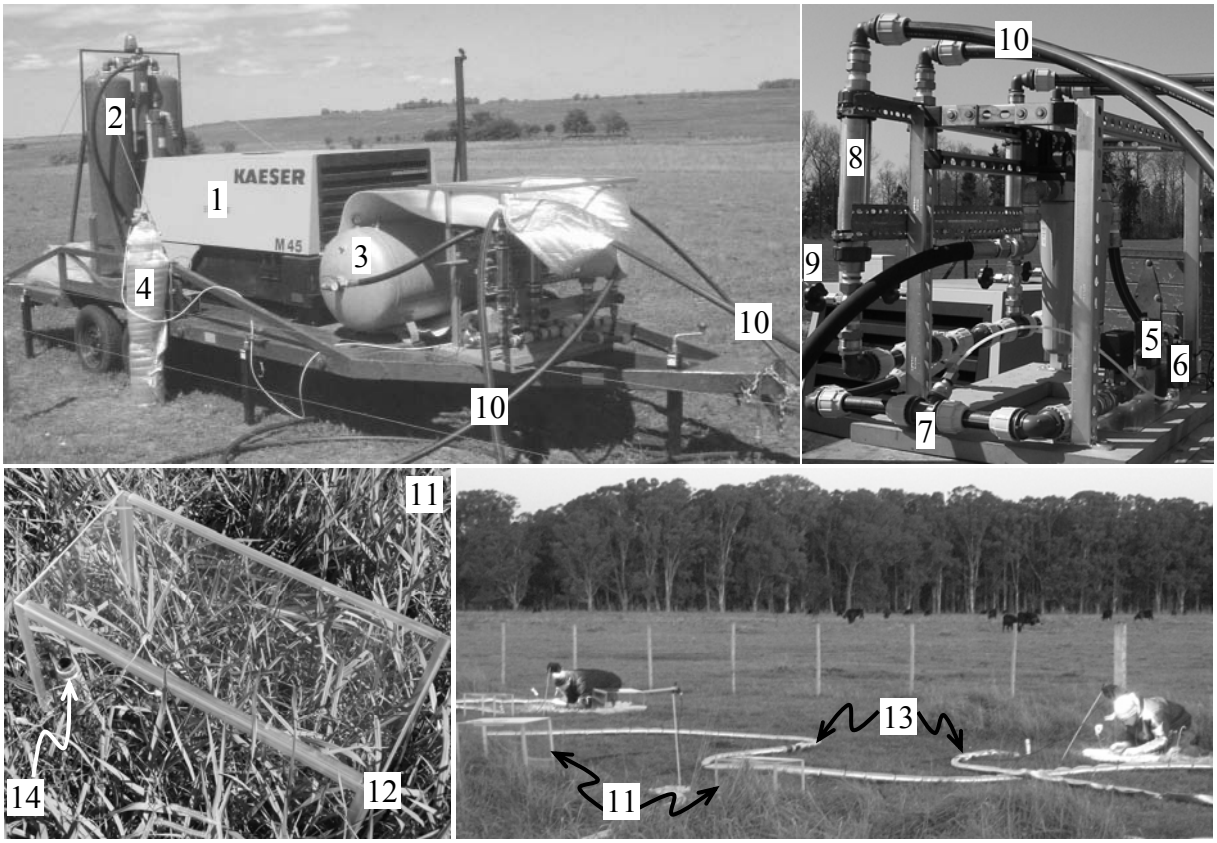

Fig. II.1. Labeling facility comprising (1) compressor, (2) adsorption dryer for CO_2 and H_2O removal, (3) buffer tank, (4) cylinder with pure CO_2, including pressure regulator, (5) mass flow controller for CO_2-free air, (6) mass flow controller for pure CO_2, (7) mixing point, (8) one of the four optical flow meters, (9) one of the four needle valves, (10) pipeline (to be buried), (11) labeling chamber made of acrylic panels held together with (12) corner profiles made of transparent polycarbonate. (13) End-pipelines wrapped in thermal insulation (14) entry point of pipeline into labeling chamber (from Lattanzi, Berone, et al. *in press*)

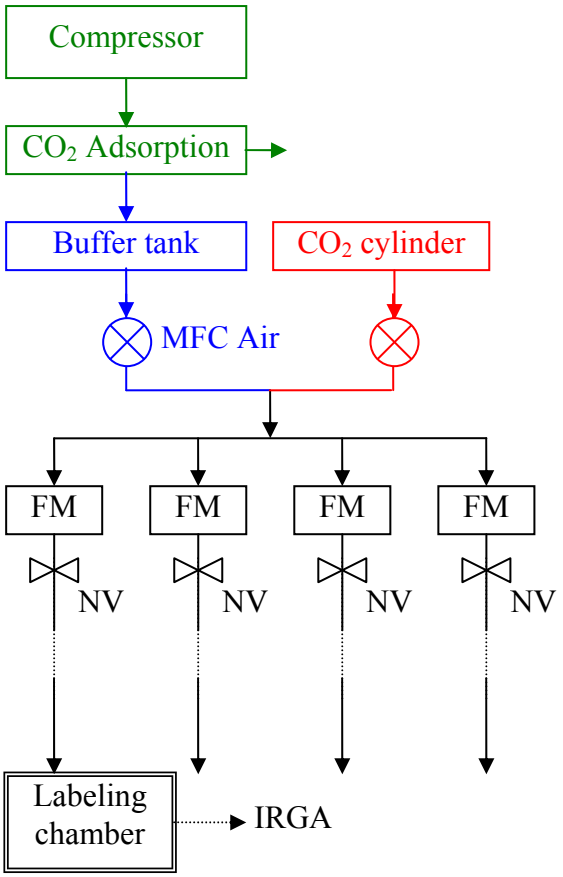

Fig. II.2. Scheme of labeling facility. MFC: digital adjustable mass flow controller, FM: optical flow meter, NV: needle valve, IRGA: infrared gas analyzer for monitoring of pCO_2 inside the labeling chamber. Arrows indicate flow of atmospheric air (green), CO_2-/H_2O-free air (blue), pure CO_2 (red), and mixed, labeling air (black) (from Lattanzi, Berone, et al. *in press*).

II.2 Quantification of tracer loss from shoots during the labeling period

^{13}C content of shoots is an unbiased measurement of gross carbon gain if no tracer is lost from shoots during the labeling period. To assess the validity of this assumption, the $\delta^{13}C$ of root and soil organic carbon was followed over nine hours of steady state labeling in two experiments. Further, the fraction of tracer lost from shoots (f_{lost}) during eight hours of steady state labeling was quantified in controlled studies carried out at our laboratory (Lehmeier et al. 2008; 2010a; 2010b) using a facility with which tracer respired and partitioned belowground can be accurately quantified (Schnyder et al. 2003; Lötscher et al. 2004).

Furthermore, the 'approximate' f_{lost} in two field studies was estimated (Gregory and Atwell 1991; Watanabe et al. 2004). 'Approximate', because shoot and/or root respiration were not measured and thus f_{lost} is underestimated. Since respiration rarely accounts for more than 50% of photosynthesis, less than half of it occurs during daylight, and the proportion of respired carbon derived from same-day assimilation is less than 20% (Lötscher, et al. 2004), the amount of tracer lost *via* respiration over one light-period would typically be less than 5 % ($0.5 \cdot 0.5 \cdot 0.2$). In consequence, true tracer loss in these studies would have been some 0.05 units higher than estimated.

Finally, another set of labeling studies (reviewed by Kuzyakov and Gavrichkova 2010) allowed us to estimate the tracer respired belowground (soil + roots) over the initial six hours after a labeling pulse, as a proportion of the total amount of tracer respired over the complete chase period.

II.3 Experimental sites

Experiments 1 to 7. Three experiments were carried out in April 2007, at the Grünschwaige Grassland Research Station of the Technische Universität München, in Germany (48º22'N, 11º50'E), in a grassland dominated by *Lolium perenne* and *Poa pratensis*, with minor amounts of *Dactylis glomerata* and *Festuca pratensis* (all C3 grasses). Rosette dicots comprised *Taraxacum officinalis* and, to a lesser extent, *Potentilla reptans*. Then, the labeling facility was shipped to Argentina, where four additional experiments were carried out in March 2008, at the Estación Experimental Balcarce, of the Instituto Nacional de Tecnología Agropecuaria (INTA) in the SE of the Pampa region (37º45'S, 58º18'W), in a grassland dominated by the grasses *L. perenne* (C3) and *Paspalum dilatatum* (C4), with minor amounts of *F. arundinacea*

(C3) and *Sporobolus indicus* (C4). Rosette dicots included *T. officinalis* and *Leontodon taraxacoides*.

Experiments 8 to 14. Twin experiments were established at a subtropical and a temperate site of the Río de la Plata grasslands. The subtropical site was located at the Estación Experimental 'Bernardo A. Rosengurtt', of the Facultad de Agronomía, Universidad de la República, in the República Oriental del Uruguay (31°25'S, 57°55'W, Fig. II.3). Mean temperature of the coldest and warmest months are 12 °C and 25 °C, respectively (Fig. II. 4), with an annual mean of 18 °C (average of maximum 24 °C, average of minimum 13 °C; frost-free period of 270 days from September to May). Monthly precipitation ranges from 70 mm to 100 mm in winter, and from 120 mm to 160 mm in spring, summer and autumn, with an average total of 1320 mm per year. The general topography of the area is flat, with basaltic soils as the dominant type.

The temperate site was located at the Campo Experimental 'Colonia Ortiz Basualdo' of the Estación Experimental Cuenca del Salado, of the Instituto Nacional de Tecnología Agropecuaria (INTA), in the República Argentina (37°05'S, 57°52'W, Fig. II.3). Mean temperature for the coldest and warmest months are 8 °C and 21 °C respectively (Fig. II. 4), with an annual mean of 14 °C (average of maximum 20 °C, average of minimum 8 °C; frost-free period of 210 days from October to April). Precipitation ranges between 30 mm and 60 mm per month in winter, and 70 mm and 120 mm in spring, summer and autumn, with a mean total of 930 mm per year. The general topography of the area is flat, and soils are mainly humic gley soils, with some alkalinity in subsuperficial horizons.

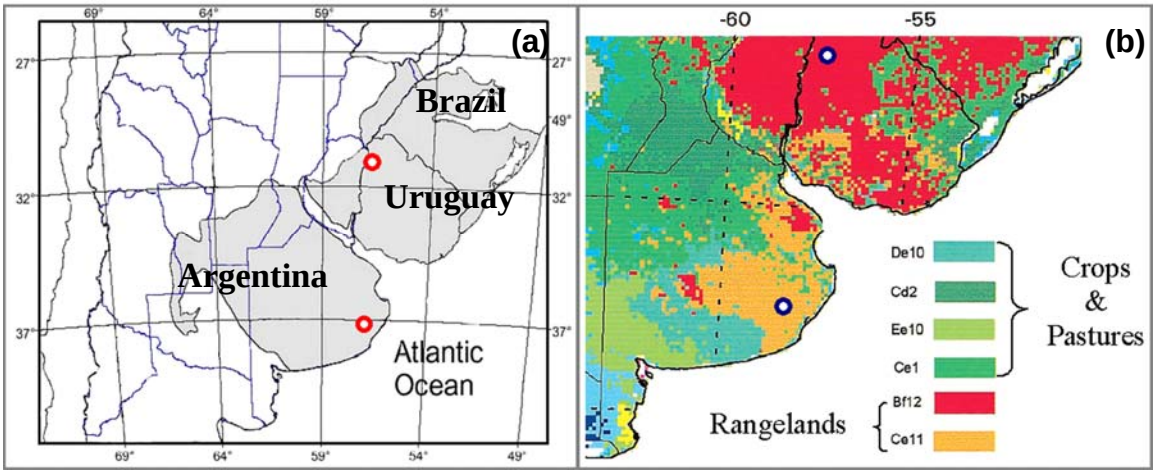

Fig. II.3. (a) Original extent and (b) present-day land use of the Río de la Plata grasslands, in South America (after Soriano 1991; Paruelo et al. 2001). Circles show the location of the two sites involved in the present thesis, the temperate in Argentina, and the subtropical in Uruguay (the distance between these sites is approx. 1000 km).

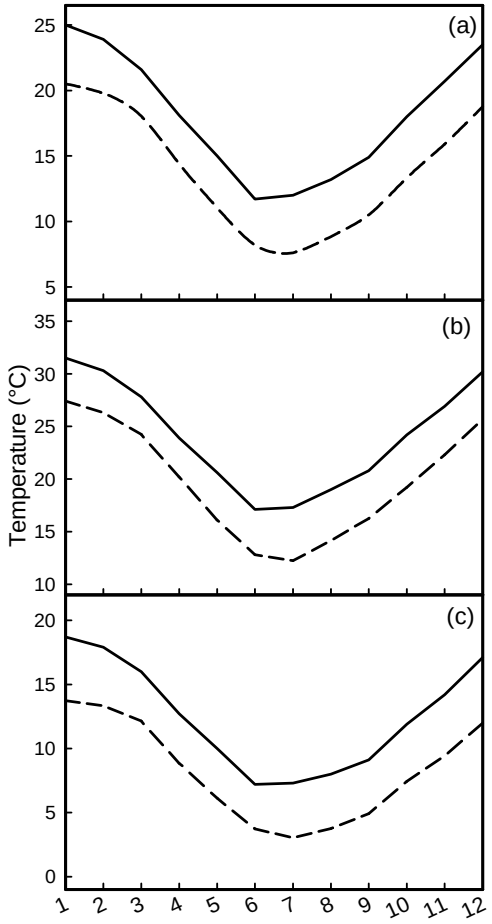

Fig. II.4. Historical averages of mean (a), maximum (b) and minimum (c) air temperature for the temperate (dashed line) and the subtropical (continuous line) site evaluated. 1 = January, 12 = December.

II.3.1 Defoliation management in Experiments 8 to 14

The aim of grazing management was to minimize above ground plant-plant interactions, ensuring that the majority of shoots were well-lit (Fahnestock and Knapp 1993; Werger et al. 2002). For this, swards were kept at a constant sward surface height of around 0.10 m, which corresponded to an herbage biomass of 100 to 150 g dry matter/m^2, and a LAI of 1.0 to 1.5 m^2 of green blade per m^2 of ground soil, throughout the year *via* continuous grazing of heifers with frequent adjustments of the stocking rate. Stocking rates were adjusted following a put-and-take protocol, based on weekly measurements of sward surface height as described in Agnusdei and Mazzanti (2001) and Schnyder et al. (2006).

II.4 Labeling conditions

All the experiments were carried out on clear, sunny days. Sampled places were irrigated, if necessary, to maintain a high soil water content over the two-weeks period before the day on which labeling was actually performed. Chambers were placed on the stands without any attachment to the ground, hence enclosed and surrounding vegetation were disturbed only minimally (Fig. II.1). To minimize shadows inside the chamber, they were disposed with the longer side aligned on a East-West axis, and the inlet tube located at the Northern side (Germany) or the Southern side (Argentina and Uruguay).

In Exps. 1, 3 and 5, chambers were distributed within a strip of 20 m · 60 m (*i.e.* ~0.12 hectares). In Exps. 2, 4, 6 and 7, chambers were located within an area of 20 m · 30 m (*i.e.* ~0.06 hectares). In Exps. 8 – 14, chambers were distributed over grazed plots of approximately 2 hectares, within a strip of 50 m · 100 m (*i.e.* ~0.5 hectares) – a limit imposed by the length of available pipeline. In all cases, chambers

were located on areas rich in the sampled species, avoiding places with recent depositions of urine or feces (Fig. II.5).

Immediately after placement, each chamber received air at a rate of 90 L min^{-1} (Exps. 1 to 7) and at a rate of 65 L min^{-1} to 85 L min^{-1} (Exps. 8 to 14). The pCO_2 was kept at between 360 and 380 µbar. Table II.1, details the experimental conditions (*e.g.* labeling duration, canopy and climate conditions, species sampled, etc).

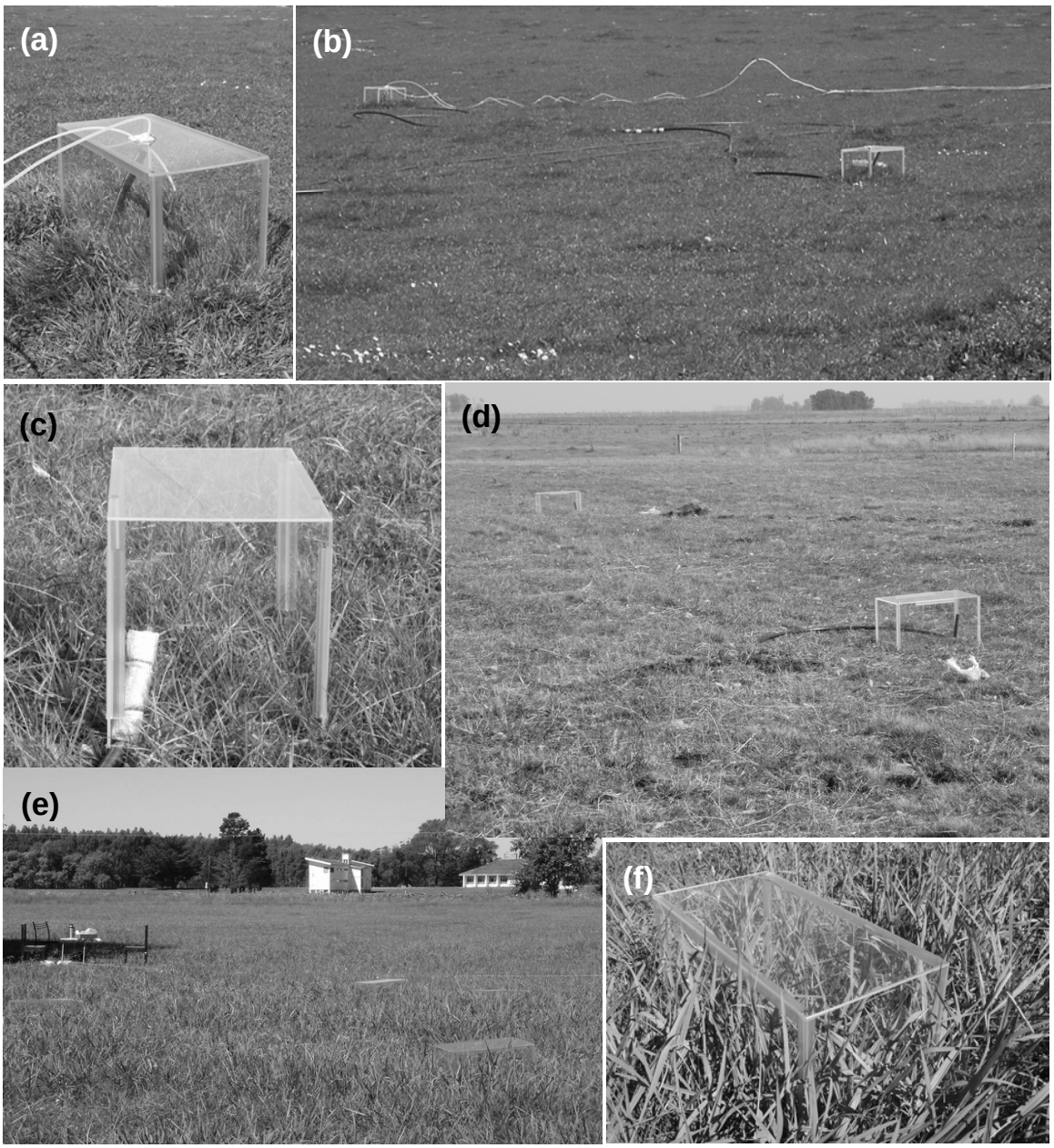

Fig. II.5. Views of labeling chambers during Exp. 1 (a, b), Exp. 8 (c, d), and Exp. 2 (e, f).

Table II.1. Stand characteristics and climatic conditions during the labeling period, for each experiment.

Location	Germany	Argentina (Balcarce)			Argentina (Colonia Ortiz Basualdo)					Uruguay	
Experiment [a]	1, 3, 5	2, 6	4	7	8	9	10	11	12	13	14
Season	Early spring	End of summer			Winter 'cooler day'	Winter 'typical day'	Winter 'warmer day'	Autumn 'typical day'	Autumn 'typical day'	Winter 'typical day'	Autumn 'warmer day'
Date (d-m-y)	11-4-07	15-3-08			8-7-09	8-8-08	15-7-08	8-5-08	08-5-09	1-7-09	22-5-09
Stand height, m	0.12	0.28		0.26	0.11	0.10	0.10	0.13	0.11	0.11	0.12
Stand biomass, g d.wt. m^{-2}	402	445		283	102	112	131	131	124	107	126
Stand leaf area index	3.7	4.9		3.1	0.9	1.0	1.1	1.2	1.1	1.1	1.2
Morphological measurements	No	No	Yes	No	Yes [f]	Yes	Yes	Yes	Yes [f]	Yes [f]	Yes [f]
Labeling period	10 to 19 hs	10 to 19 hs	12:20 to 19 hs	10:30 to 19 hs	9:40 to 16:00 hs	9:20 to 16:30 hs	9:30 to 17:00 hs	10:00 to 17:00 hs	10:10 to 17:00 hs	9:40 to 16:00 hs	9:20 to 16:30 hs
Daily PAR [b], MJ m^{-2} d^{-1}	7.7	9.2	9.2	9.2	4.0	5.3	3.7	4.7	4.8	4.3	5.1
Fraction of daily PAR accumulated	0.90	0.90	0.70	0.86	0.91	0.92	0.92	0.92	0.86	0.89	0.89
Mean PAR during labeling [b], µmol m^{-2} s^{-1}	1025	1308	1324	834	694	667	654	763	775	745	874
Mean air temperature during labeling [c], ºC	21.3	25.7	26.9	20.2	7.6	14.3	24.0	22.0	23.1	23.3	29.8
Difference in temperature [d], ºC	- 0.91	- 0.60	- 0.79	no data	-1.4	3.0	2.2	2.3	3.1	2.0	2.6
Mean daily temperature [b], ºC	10.3	19.1	19.1	19.1	5.2	9.6	15.5	12.1	12.5	11.5	22.1
Historical mean daily temperature [b], ºC	9.5	18.0	18.0	18.0	8.2	8.2	8.2	11.1	11.1	12.0	15.0
C3 species sampled [e]	Lp, Pp, Rd	Lp, Rd			Bu, Sp	Bu, Sp, Lm, Rd	Bu, Sp, Lm, Rd	Bu, Sp, Rd	Bu, Sp	Ba, Ss, Pp	Ba, Ss, Pp
C4 species sampled	absent	Pd			Pd	Pd	Pd	Pd, Bl	Pd, Bl	Pn, Bl, Cs	Pn, Bl, Cs

[a] Exps. 1 and 2: analysis of the time-course of tracer incorporation in above- and below-ground biomass (Chapter III.1), Exps. 3 to 7: analysis of carbon gain of individuals and functional groups (Chapter III.2), Exps. 8 to 14: analysis of carbon gain in C3 and C4 species (Chapter III.3). [b] outside chamber. [c] inside chamber. [d] inside chamber minus outside chamber. [e] (Lp) *Lolium perenne*; (Pp) *Poa pratensis*; (Bu) *Bromus unioloides*; (Sp) *Stipa papposa*; (Ba) *B. auleticus*; (Ss) *S. setigera*; (Pp) *Pitochaetium* sp.; (Lm) *L. multiflorum*; (Rd) Rosette dicots; (Pd) *Paspalum dilatatum*; (Bl) *Bothriochloa laguroides*; (Pn) *P. notatum*; (Cs) *Coelorachis selloana*. [f] made on the same plots, but not on the labeled tillers (see Materials and Methods). d.wt.: dry weight

II.5 Sample collection

Experiments 1 and 2: evolution of $\delta^{13}C$ in shoots and in belowground organic carbon

Soil cores (depth: 0.2 m; Ø: 0.05 m) and shoots of C3 and C4 species were sampled at few hours intervals over 9 h of steady state labeling (Exp. 1: one chamber; Exp. 2: two chambers). Soil and shoots sampled outside the chambers served as unlabeled controls for ^{13}C content.

Experiments 3 and 4: relationship between the carbon gain and size of individuals

Coexisting individuals of contrasting size –defined, for grasses, as tillers holding at least three mature leaves, and as individual rosettes for the dicots species– were harvested after either 9 h (Exp. 3) or 6 h (Exp. 4) of steady state labeling (Exp. 3: 41 tillers of *L. perenne*, 16 of *P. pratensis*, and 9 *T. officinalis* rosettes; Exp. 4: 27 tillers of *L. perenne*, 25 of *P. dilatatum*, and 11 *T. officinalis* rosettes). All individuals were vegetative. Three chambers were used in each experiment.

Experiments 5, 6, and 7: carbon gain of functional groups

Standing biomass of 0.04 m^2 quadrates was harvested after labeling (n=1 in Exp. 5, n=2 in Exps. 6 and 7), and immediately separated into rosette dicots, C3 grasses, and C4 grasses (absent in Exp. 5). Dead tissue was discarded. No legume was present. Labeling chambers were located either in open, sunny places (Exps. 5 and 6) or shaded by trees (Exp. 7).

Experiments 8 to 14: carbon gain of C3 and C4 species

In each experiment, individuals of the most frequent species were harvested (Appendix provides a brief description of each sampled species). The complete range

of shoot sizes present in the field was sampled. In experiments 9, 10 and 11, 10 to 20 shoots per species were harvested and individually analyzed. In experiments 8, 12, 13 and 14, 20 to 40 shoots were harvested and clustered according to size in 3 to 5 groups comprising 4 to 6 tillers each.

II.6 Leaf area measurements and allometry

In Exps. 4, 9, 10 and 11, individuals were photographed after harvest on a graduated board (Figure II.6). Images were analyzed with "Assess" (v 2.0; Lamari 2008) to estimate green leaf area (blade area, in grasses), leaf length (blade plus sheath in grasses), and extended shoot height (*i.e.* the length of the longest leaf). No difference was observed in leaf area measured *via* image analysis or with a leaf area meter (LI 3100, LI-COR, Lincoln, USA. Data not shown).

Allometric equations of individual's leaf area *vs.* carbon mass were obtained for each site, species and season. The proportionality between blade area produced per unit shoot carbon mass and individual's size (that is, the value of the scaling exponent) was tested on regressions fitted to log-transformed data using standard major axis regression, which accounts for error in both the *x*- and the *y*-axis (Warton et al. 2006). However, the equations used to predict the LAR of each individual from its mass were fitted to untransformed data using normal least-squares regression, which only accounts for error in the *y*-axis (Warton et al. 2006). In Exps. 9, 10 and 11 this analysis was carried out using the labeled individuals, but in Exps. 8, 12, 13 and 14, the analysis was carried out in non-labeled individuals sampled from the same plots used during labeling.

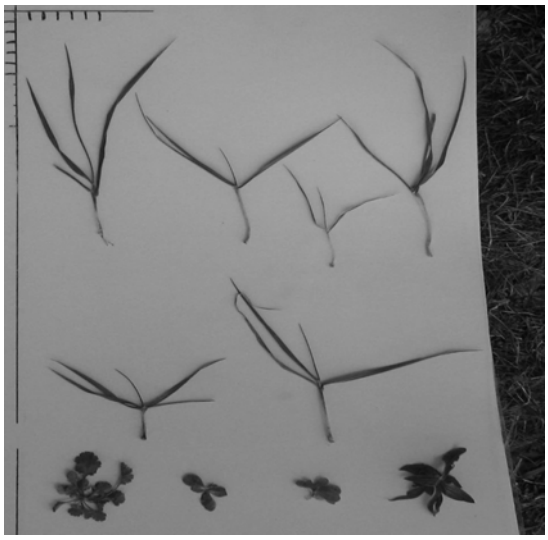

Fig. II.6. Individuals of *Bromus unioloides* and rosette dicots photographed after harvest (Exp. 9) on a graduated board.

II.7 Sample processing

In Exps. 1 to 7, samples were dried immediately after harvest at 60°C for 72 h. In Exps. 8 to 14, samples were frozen in liquid nitrogen immediately after harvest (*i.e.* in the field), stored at -20 °C, and then freeze-dried (Rificor LA-B4, Rificor SH, Buenos Aires, Argentina). For all the experiments, dry samples were weighted and milled to a fine powder with a ball mill (MM200, Retsch, Muenchen, Germany).

II.8 Analysis of carbon isotopic composition

Carbon and nitrogen content, and the ratio of ^{13}C to ^{12}C isotopes, were determined on 0.7 mg dry matter using an elemental analyzer (NA1500, Carlo Erba Strumentazione, Milan, Italy) interfaced to a continuous flow isotope mass ratio spectrometer (Delta^{plus}, Finnigan MAT, Bremen, Germany). Samples were measured against a working gas standard previously calibrated against a secondary isotope standard (IAEA-CH6, accuracy ±0.06 ‰ SD). A laboratory standard (wheat flour) was run after every tenth sample to estimate the precision of the isotope analyses (±0.09 ‰ SD).

II.9 Estimation of the daily carbon gain and its components

Daily carbon gain per unit shoot mass, referred to as relative gross photosynthesis rate (RPR), was estimated as the amount of tracer accumulated over the labeling period multiplied by the ratio of daily PAR (PAR_{day}) to PAR accumulated over the labeling period (PAR_{lab}). This assumed a linear relationship between cumulative PAR and tracer gain (supported by results, see below). RPR (d^{-1}) was analyzed as the product of leaf area per unit shoot carbon mass (LAR, $m^2\ g^{-1}$) and carbon gained per unit leaf area (GAR, $g\ m^{-2}\ d^{-1}$). LAR was estimated from the allometric relationship between leaf area and mass, and GAR, as RPR divided by LAR.

Tracer content was estimated from the ^{13}C content of labeled plants (S), of unlabeled plants (U), and of newly assimilated CO_2 (L). Thus, RPR was estimated as:

$$RPR = (S - U) / (L - U) \cdot (PAR_{day}/PAR_{lab}) \qquad (1)$$

Since variation in natural $\delta^{13}C$ abundance between individuals with different hierarchical position, and between organs within an individual, can be large (Schnyder and Lattanzi 2005), care was taken in sampling labeled and unlabeled individuals with similar morphology.

L was estimated assuming that ^{13}C discrimination during CO_2 assimilation (Δ) was the same inside and outside the labeling chambers,

$$L = (\delta^{13}C_{cham} - \Delta) / (1000 + \Delta) \cdot 1000 \qquad (2)$$

$$\Delta = (\delta^{13}C_{atm} - U) / (1000 + U) \cdot 1000 \qquad (3)$$

where $\delta^{13}C_{cham}$ is the ^{13}C content of CO_2 inside the chambers (assumed 1.5‰ lower

than that of the pure CO_2), and $\delta^{13}C_{atm}$ is the ^{13}C content of atmospheric CO_2 (assumed –8.2‰).

II.10 Statistical analyses

Experiments 1 to 7. The evolution of $\delta^{13}C$ in shoots and belowground carbon during the labeling period in Exp. 2 was analyzed by repeated measures ANOVA in a completely random design (PROC MIXED with a first-order autoregressive structure, SAS v9.1, SAS Institute, Cary, USA). Labeling chambers were the experimental unit on which the repeated measures were taken. Exp. 1 had one replication; no statistical analysis was performed. Relationships between RPR and individual's size (Exps. 3 and 4) were analyzed by regression. Non-linearity was tested using Akaike's Information Criterion (Motulsky and Christopoulos 2003). Standing biomass and RPR of functional groups (Exps. 5, 6 and 7) were analyzed by ANOVA in a completely random design (PROC GLM, with 'experiment' and 'functional group' as classification variables). Each quadrate was considered an experimental unit, independently of in which labeling chamber it was located. Standing biomass (Exp. 7) was log-transformed to account for variance heteroscedasticity.

Experiments 8 to 14. Confidence intervals were estimated for RPR, GAR and LAR. SEM were derived from the root mean square of the error of ANOVAs run for each 'site' and 'season' using a completely random design with 'species' as the classification variable (PROC GLM, SAS v.9.1, SAS Institute, Cary, USA). Data were checked for homogeneity of variances within each 'site' and 'season' using the Levene test.

Experimental unit. In all experiments dealing with carbon gain of individuals (Exps. 3, 4, 8, 9, 10, 11, 12, 13 and 14), each individual was considered an experimental unit, independently of which labeling chamber it was located, Thus, it was assumed that there were no intrinsic chamber effects on carbon gain. This was checked in the three species sampled in Exp. 4: no intrinsic chamber effect was detected (Fig. II.7).

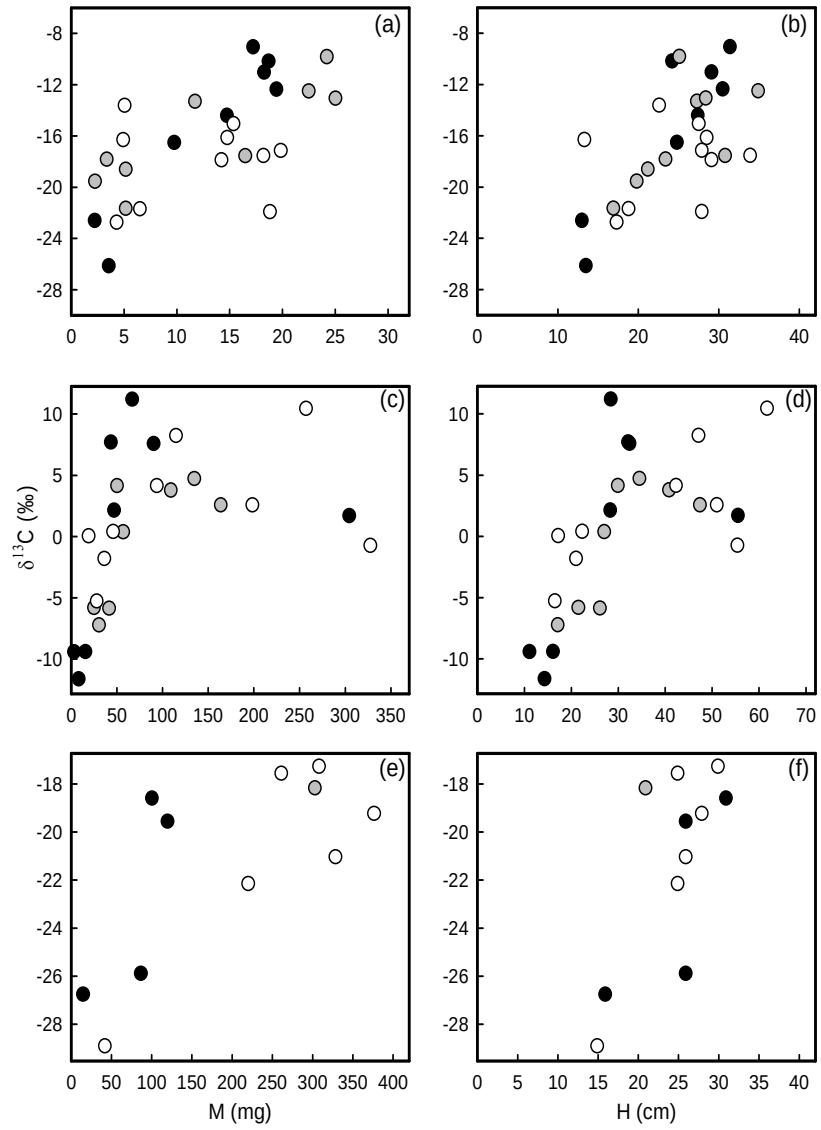

Fig. II.7. $\delta^{13}C$ as a function of shoot mass (M; a,c,e) and extended shoot height (H; b,d,f) of individuals of *Lolium perenne* (a,b), *Paspalum dilatatum* (c,d) and *Taraxacum officinalis* (e,f) sampled in chamber 1 (black circles), chamber 2 (grey circles) and chamber 3 (white circles), in a grassland of Argentina (Exp. 4).

III. RESULTS

Following the general structure of this thesis, results are presented in three separate sections: the proportion of tracer lost from shoots during labeling are evaluated first (section III.1), then the daily carbon gain of individuals and functional groups growing in dense field stands differing in canopy structure are shown (section III.2), and finally the results of daily carbon gain in C3 and C4 species growing in mixed natural grasslands, during the autumn-to-winter transition are examined (section III.3).

III.1 Assessment of the assumption of no tracer loss (Exps. 1 and 2)

The ^{13}C content of a shoot is an unbiased measurement of its gross carbon gain if no tracer is lost during the labeling period. To assess the validity of this assumption, the $\delta^{13}C$ of shoots and of belowground organic carbon were followed over nine hours of steady state labeling. $\delta^{13}C$ of shoots increased near linearly with time and with cumulative PAR ($r^2 > 0.95$, Fig. III.1). Conversely, the $\delta^{13}C$ of root plus soil organic carbon did not change within the initial six hours of labeling (Fig. III.1). Afterwards, $\delta^{13}C$ of belowground carbon increased slowly.

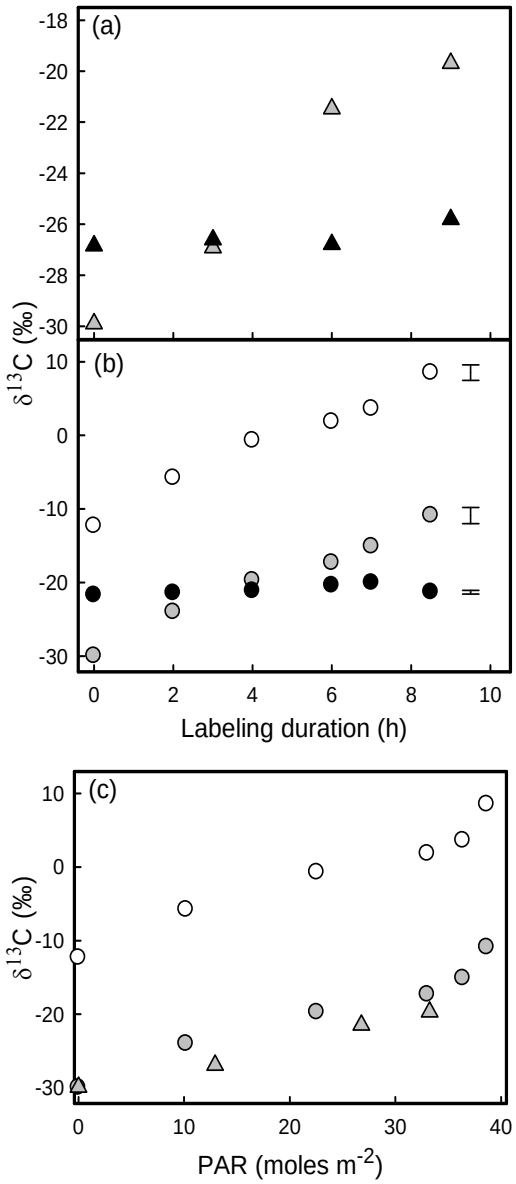

Fig. III.1. Evolution of the $\delta^{13}C$ of shoots of C3 (grey symbols) and C4 species (white symbols) and of belowground organic carbon (0 – 0.2 m: roots plus soil, black symbols) during nine hours of steady state ^{13}C labeling in grasslands of (a) Germany (Exp. 1) and (b) Argentina (Exp. 2). Bars indicate SEM (n=2). Evolution of the $\delta^{13}C$ of shoots of C3 (grey symbols) and C4 species (white symbols) as a function of cumulative PAR in grasslands of Germany (triangles) and Argentina (circles) (c) (from Lattanzi, Berone, et al. *in press*).

A survey of data in the literature revealed that after eight h of steady state labeling, f_{lost} in *L. perenne* plants grown under a 16/8 h day/night cycle was 0.13. Plants in continuous light had a higher f_{lost}, particularly if they were nitrogen limited (Table III.1). Likewise, in field crops f_{lost} ranged from 0.03 to 0.13 in mature plants,

but was higher than 0.30 in seedlings (Table III.1). Finally, results from pulse-chase labeling studies showed that less than 9 % of all tracer respired was respired during the initial six hours of the chase period (Table III.2).

Table III.1. Fraction of tracer lost from shoots (f_{lost}) after eight hours of steady state labeling in five independent studies.

Species	Growth conditions, and stand developmental stage	f_{lost}
Lolium perenne [a]	Continuous light, high nitrogen supply. Vegetative	0.17
Lolium perenne [b]	Continuous light, low nitrogen supply. Vegetative	0.22
Lolium perenne [c]	16 h/8 h light/dark, high nitrogen supply. Vegetative	0.13
Triticum aestivum [d]	Field, 50 DAS. Seedlings (84 g d.wt. m^{-2})	0.34*
Triticum aestivum [d]	Field, 71 DAS. Vegetative (218 g d.wt. m^{-2})	0.12*
Triticum aestivum [d]	Field, 106 DAS. Anthesis (673 g d.wt. m^{-2})	0.06*
Triticum aestivum [d]	Field, 120 DAS. Grain filling (705 g d.wt. m^{-2})	0.06*
Hordeum vulgare [d]	Field, 49 DAS. Seedlings (37 g d.wt. m^{-2})	0.36*
Hordeum vulgare [d]	Field, 70 DAS. Vegetative (119 g d.wt. m^{-2})	0.13*
Hordeum vulgare [d]	Field, 105 DAS. Anthesis (522 g d.wt. m^{-2})	0.02*
Hordeum vulgare [d]	Field, 126 DAS. Grain filling (374 g d.wt. m^{-2})	0.03*
Oryza sativa [e]	Field, 47 DAT. Vegetative	0.05**
Oryza sativa [e]	Field, 76 DAT. Booting	0.02**
Oryza sativa [e]	Field, 106 DAT. Grain filling	0.01**

*Shoot respiration was not measured **Nor shoot nor root respiration were measured, please see Material and Methods (p. 15) for details. [a]Lehmeier et al. (2008), [b] Lehmeier et al. (2010b), [c] Lehmeier et al. (2010a), [d] Gregory and Atwell (1991), [e] Watanabe et al. (2004). DAS: days after sowing, DAT: days after transplant, d.wt.: dry weight.

Table III.2. Tracer respired belowground (soil + roots) over the initial six hours after a labeling pulse, as a proportion of total tracer respired during the complete chase period.

Species	Growth conditions	Observations	
Triticum aestivum [a]	Field	Elongation stage	0.03
Triticum aestivum [a]	Field	Ear emergence stage	0.03
Triticum aestivum [a]	Field	Anthesis stage	0.01
Triticum aestivum [a]	Field	Milk ripening stage	0.01
Triticum aestivum [a]	Field	Dough ripening stage	0.01
Bromus erectus [b]	Greenhouse	Vegetative plants, poor soil	0.03
Bromus erectus [b]	Greenhouse	Vegetative plants, rich soil	0.06
Lolium perenne [c]	Growth chamber	Vegetative plants, 35 cm height	0.11
Lolium perenne [c]	Growth chamber	Vegetative plants, 19 cm height	0.14
Zea mays [d]	Growth chamber	Vegetative plants	0.13
Lolium perenne [e]	Growth chamber	Vegetative plants, 23 cm height	0.12
Lolium perenne [e]	Growth chamber	Vegetative plants, 32 cm height	0.11
Lolium perenne [f]	Growth chamber	Vegetative plants	0.15
Lolium perenne [g]	Growth chamber	Vegetative plants	0.14
Lactuca sativa [h]	Growth chamber	Vegetative plants, high nitrogen	0.12
Lactuca sativa [h]	Growth chamber	Vegetative plants, low nitrogen	0.14

[a] Fig. 3 in Swinnen et al. (1994); [b] Fig. 1 in Warembourg and Estelrich (2000); [c] Fig. 1 in Kuzyakov et al. (1999); [d] Fig. 6 in Nguyen et al. (1999); [e] Fig. 3 in Kuzyakov et al. (2001); [f] Fig. 1 in Domanski et al. (2001); [g] Fig. 4 in Kuzyakov and Domanski (2002); [h] Fig. 5 in Kuzyakov et al. (2002)

III.2 Carbon gain of individuals and functional groups in dense field stands

III.2.1 Carbon gain of individuals (Exps. 3 and 4)

Within each species, RPR was generally lower in small individuals. But the relationship between RPR and individual's mass became generally asymptotic (Fig. III.2).

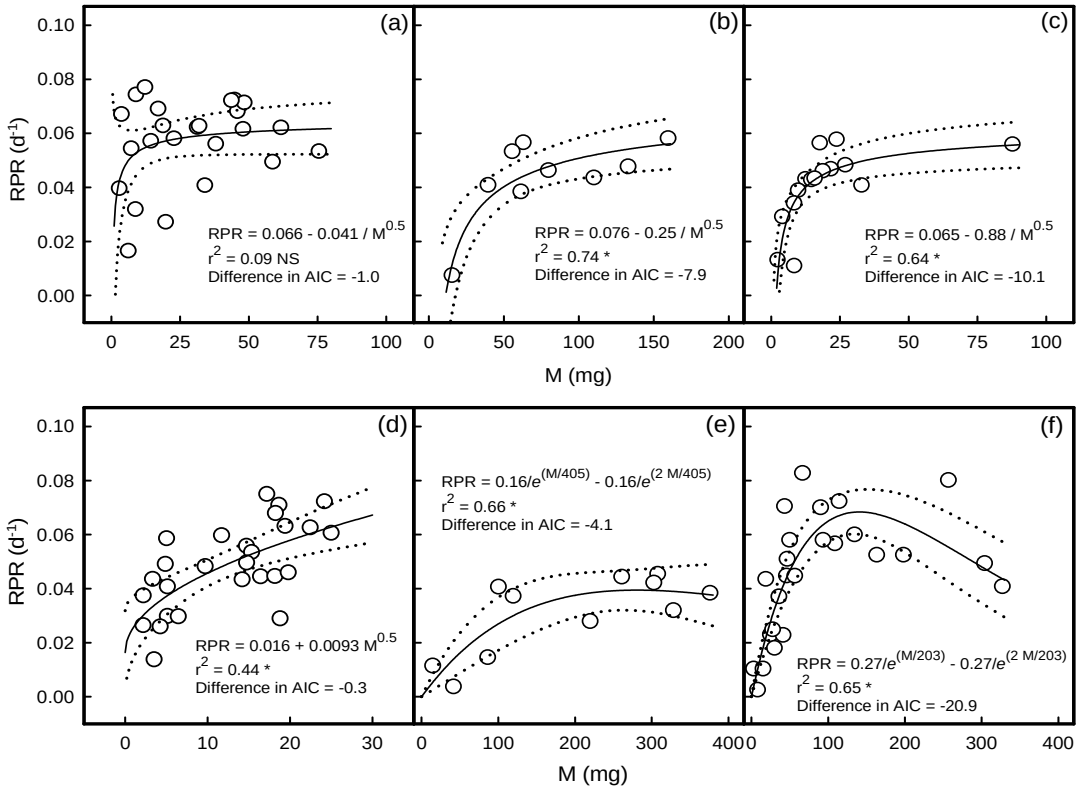

Fig. III.2. Relative gross photosynthesis rate (RPR) as a function of shoot mass (M) of individuals coexisting in relatively short (Exp. 3: a-c) or tall stands (Exp. 4: d-f). Each symbol is an individual: (a, d) *Lolium perenne*, (b, e) *Taraxacum officinalis*, (c) *Poa pratensis*, and (e) *Paspalum dilatatum*. Regression lines and 95% confidence intervals are shown. * indicates $P < 0.01$, NS indicates $P > 0.15$, and 'Difference in AIC' is the difference in Akaike's Information Criterion between nonlinear and linear models (a negative difference means the nonlinear model is better) (from Lattanzi, Berone, et al. *in press*).

In all cases, grasses showed higher RPR than rosette dicots when compared at a given shoot mass. In Exp. 4, the C3 grass *L. perenne* had higher RPR than the C4

grass *P. dilatatum*. Interspecific differences largely disappeared when RPR was plotted against extended shoot height (Fig. III.3). This resulted from contrasting mass-height allometric relationship between species (Fig. III.3), which also lessened interspecific differences in nitrogen content per unit leaf area (N_L, Fig. III.3).

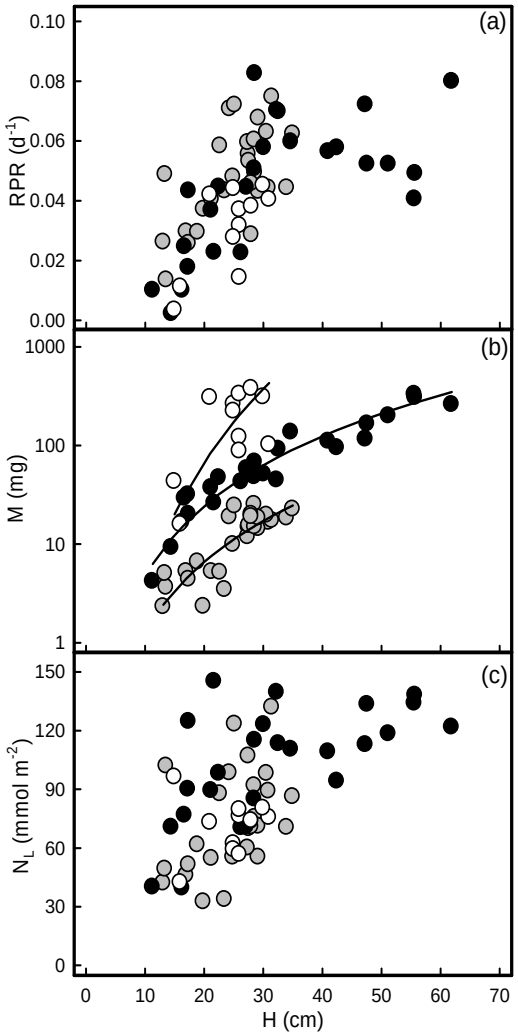

Fig. III.3. (a) Relative photosynthesis rate (RPR), (b) shoot mass (M), and (c) nitrogen content per unit leaf area (N_L) as a function of extended shoot height (H) of individuals coexisting in a stand (Exp. 4). Each symbol is an individual: *Paspalum dilatatum* (black circles), *Lolium perenne* (grey circles), and *Taraxacum officinalis* (white circles). Lines are fitted allometric relationships: $M = 0.0056 \cdot H^{2.36}$ (*L. perenne*), $M = 0.0002 \cdot H^{4.22}$ (*T. officinalis*), $M = 0.0207 \cdot H^{2.36}$ (*P. dilatatum*) (from Lattanzi, Berone, et al. *in press*).

In grasses, proportionally less blade area was produced per unit shoot carbon mass as plants got bigger, that is, the scaling exponent of the allometric relationship

between area and mass was lower than unity (β = 0.77). Consequently, a negative relationship developed between LAR and shoot mass (Fig. III.4). Conversely, in rosettes, LAR did not vary with size (scaling exponent close to one: β = 0.95). The lower RPR of small individuals in Exp. 4 was due to lower GAR, in spite of higher LAR, while the asymptotic behavior of RPR in the larger individuals of *P. dilatatum* was associated with stabilization of both GAR and LAR (Fig. III.4).

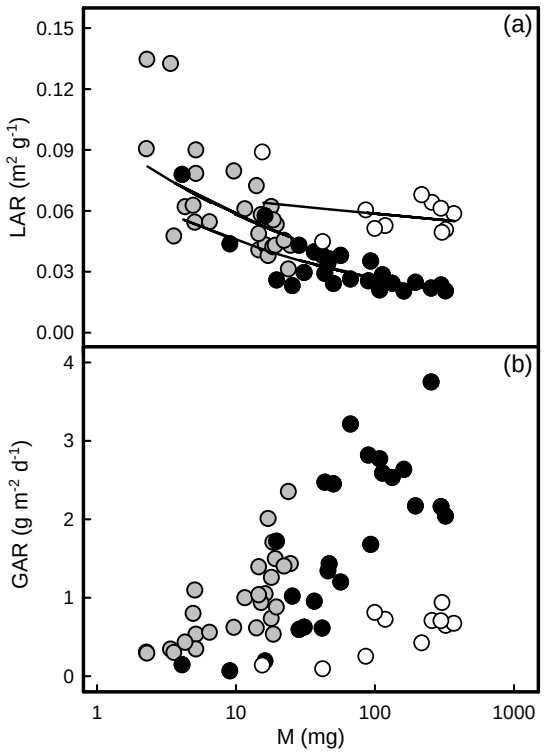

Fig. III.4. (a) Leaf area ratio (LAR) and (b) gross assimilation rate (GAR) as a function of shoot carbon mass of individuals (M) coexisting in a stand (Exp. 4). Each symbol is an individual: *Paspalum dilatatum* (black circles), *Lolium perenne* (grey circles), and *Taraxacum officinalis* (white circles). Note the logarithmic scale of the *x*-axis. Lines are fitted allometric relationships: $LAR = 0.0199 \cdot M^{0.77}$ (*L. perenne*), $LAR = 0.0525 \cdot M^{0.95}$ (*T. officinalis*), $LAR = 0.0152 \cdot M^{0.77}$ (*P. dilatatum*) (from Lattanzi, Berone, et al. *in press*).

III.2.2 Standing biomass and carbon gain of functional groups (Exps. 5, 6 and 7)

Grasses contributed more than 80 % of stand biomass in all three experiments (Fig.

III.5). C4 grasses contributed more than C3 species, both in sunny (Exp. 6) and shaded canopies (Exp. 7). However, stand dominance was a poor indicator of differences in RPR between functional groups: the RPR of C3 grasses was similar to that of rosette dicots in Exp. 5, and 1.4 and 1.9 times higher in Exps. 6 and 7, respectively. C3 and C4 grasses had similar RPR (Fig. III.5).

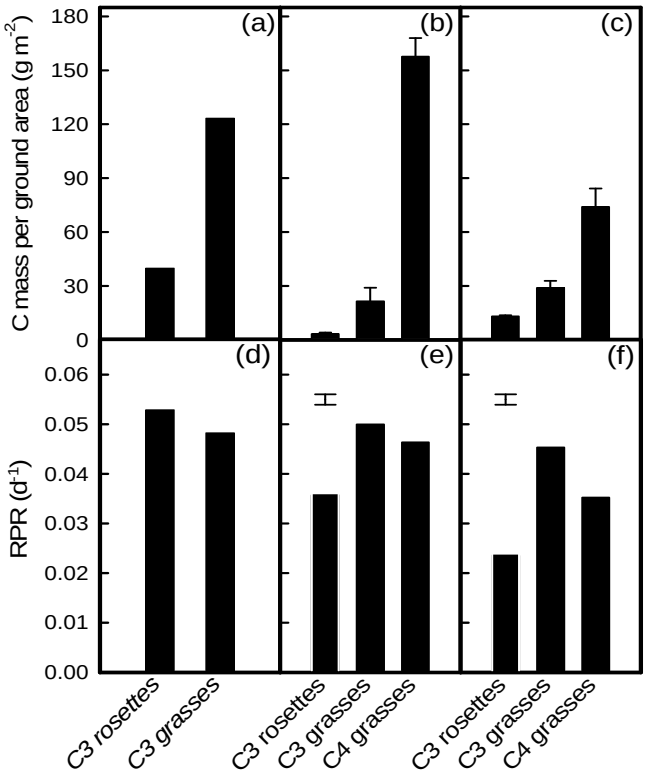

Fig. III.5. Standing mass (a-c) and relative photosynthesis rate (RPR, d-f) of rosette dicots, C3 grasses and C4 grasses in a relatively short stand (Exp. 5: a, d), a relatively tall sunny stand (Exp. 6: b, e), and a relatively tall shaded stand (Exp. 7: c, f). Bars indicate SEM (n=2 in panels b, c; n=11 in panels e, f) (from Lattanzi, Berone, et al. *in press*).

III.3 Daily carbon gain of C3 and C4 species during the autumn-to-winter transition in a temperate and a subtropical grasslands (Exps. 8 to 14)

III.3.1 Climate and canopy structure

Total incident daily PAR (MJ d^{-1}) in autumn was ~1.19 and ~1.12 times higher than that in winter in the subtropical and the temperate sites, respectively. In turn, total

incident PAR in the subtropical site was 1.02 (winter) and 1.07 (autumn) times that in the temperate site. Therefore, there was relatively little differences in total daily incident PAR in Exps. 8 to 14 (Table II.1). All PAR values were well above the saturating threshold for relative growth rate and their components as identified by Poorter and Van der Werf (1998).

In the temperate site, the temperature was similar to the historical average for Exp. 9 (winter) and Exps. 11 and 12 (autumn). But the other two experiments performed in winter experienced either unusually cold (Exp. 8) or warm (Exp. 10) conditions (Table II.1). In the subtropical site, mean daily temperature was close to the historical average for the winter labeling day (Exp. 13), and above the long-term average for the autumn experiment (Exp. 14) (Table II.1).

Standing biomass, LAI and canopy height were all low (Table II.1), as expected from the grazing management applied that aimed at reducing above-ground interactions between plants. This is reflected in a general lack of correlation between individual's size and RPR observed in all datasets (data not shown), supporting the idea that that grazing management effectively prevented the development of a hierarchy of subordinate and dominant individuals. This is important because it simplifies the interpretation of C3 *vs.* C4 responses, as these can not be ascribed to shading effects (Fahnestock and Knapp 1993; 1994; Werger et al. 2002; Aan et al. 2006; Veen (Ciska) et al. 2008).

III.3.2 Changes in RPR, GAR and LAR during the autumn-to-winter transition

At the temperate site, all species showed lower RPR in winter than autumn. The decrease was more marked in C4 than in C3 species (Fig. III.6, top panel). The seasonal reduction observed in C3 species was due to changes in GAR (Fig. III.6;

Lolium multiflorum was not measured in autumn because it was not a component of the canopy at that time of the year). Conversely, in C4 species the lower RPR was mainly due to a drastic reduction of the LAR: in *P. dilatatum* LAR explained all the seasonal variation in RPR (Fig. III.6), and in *B. laguroides* individuals had no green leaf area in winter, so that RPR was zero and their GAR could not be assessed.

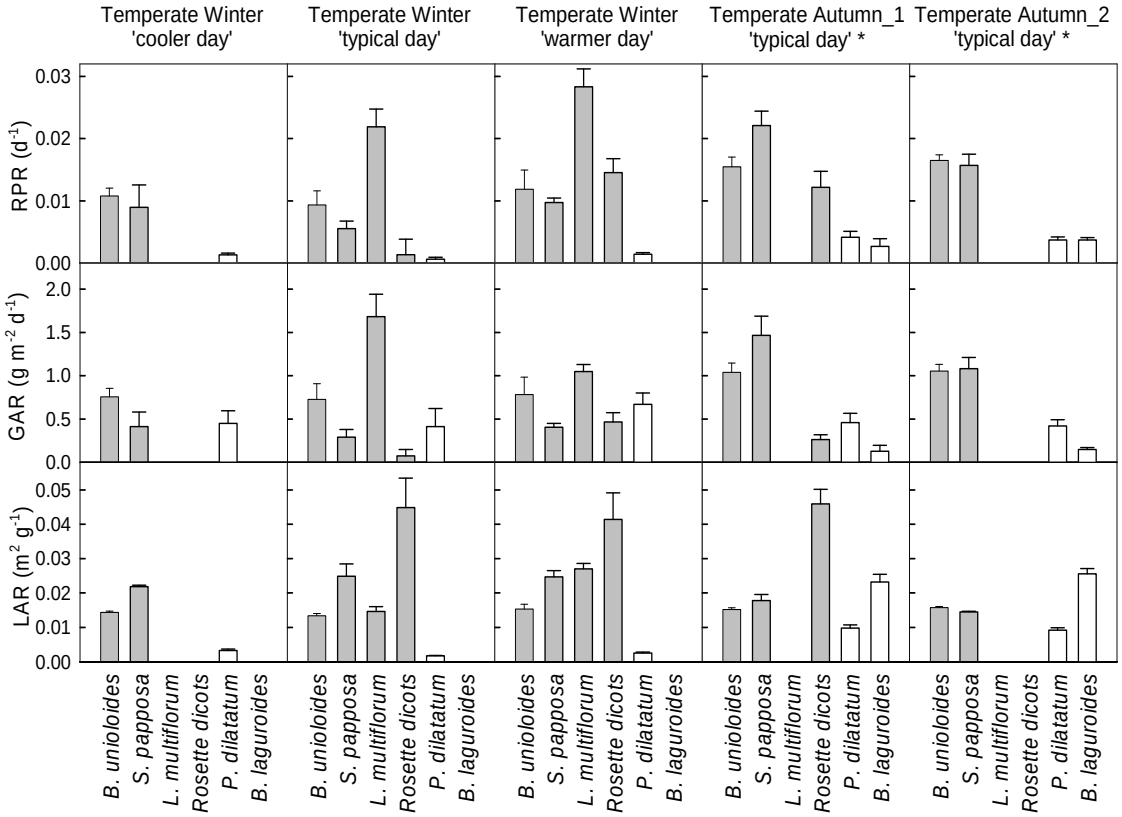

Fig. III.6. Relative photosynthesis rate (RPR, top panel), gross assimilation rate (GAR, centre panel) and leaf area ratio (LAR, bottom panel) of C3 (grey) and C4 (white) species growing at temperate site of Río de la Plata grasslands. Bars indicate SEM. * Temperate autumn_1 and Temperate autumn_2 refer to Exps. 11 and 12, respectively.

The LAR reduction observed in *P. dilatatum* was due to a reduction in blade area per tiller (Fig. III.7, middle panel), for tiller mass was only marginally lower in winter (Fig. III.7, top panel). This was due to both a lower number of green leaves per tiller, and a lower blade area per green leaf (Table III.3). In autumn, blades

represented only one third of the tiller mass, that is why the reduction in blade area per tiller over winter had a minor effect on total tiller mass (Table III.3).

Interestingly, leaves of *P. dilatatum* developed during winter had a similar GAR than those developed during the warmer days of autumn (Fig. III.6, middle panel). Indeed, the highest GAR of *P. dilatatum* was observed in the warmer winter day (Exp. 10). Although it is important to note that the age of the leaves was different between seasons, as in winter only the two youngest leaves were green (Table III.3).

Table III.3. Number of green leaves per tiller (n_L), blade area per individual leaf (A_L), specific leaf area (cm^2 of blade area per g of blade, SLA) and leaf mass proportion (g of blade per g of shoot, LWR), for grazed and non-grazed tillers of *Paspalum dilatatum* growing in autumn and winter of the temperate site. A_{L1}: growing leaf, A_{L2}: last fully expanded leaf A_{L3}: oldest leaf.

Plots	Season	n_L	A_{L1}, cm^2	A_{L2}, cm^2	A_{L3}, cm^2	SLA, $cm^2\ g^{-1}$	LWR, $g\ g^{-1}$
Grazing	Autumn 'typical day'_1 [b]	3	0.6	1.5	1.8	133	0.33
	Winter 'typical day'	2	0.4	0.7		-	-
	Winter 'warmer day'	2	0.5	0.6		-	-
Non-grazed [a]	Autumn 'typical day'_1	3	1.6	4.5	4.7	170	0.41
	Winter 'typical day'	2	0.4	0.6		-	-
	Winter 'warmer day'	2	0.4	1.0		-	-

[a] Plants growing in plots excluded from grazing during 35 and 60 days (autumn and winter, respectively). [b] Autumn 'typical day'_1 refer to Exp. 11. - Not measured

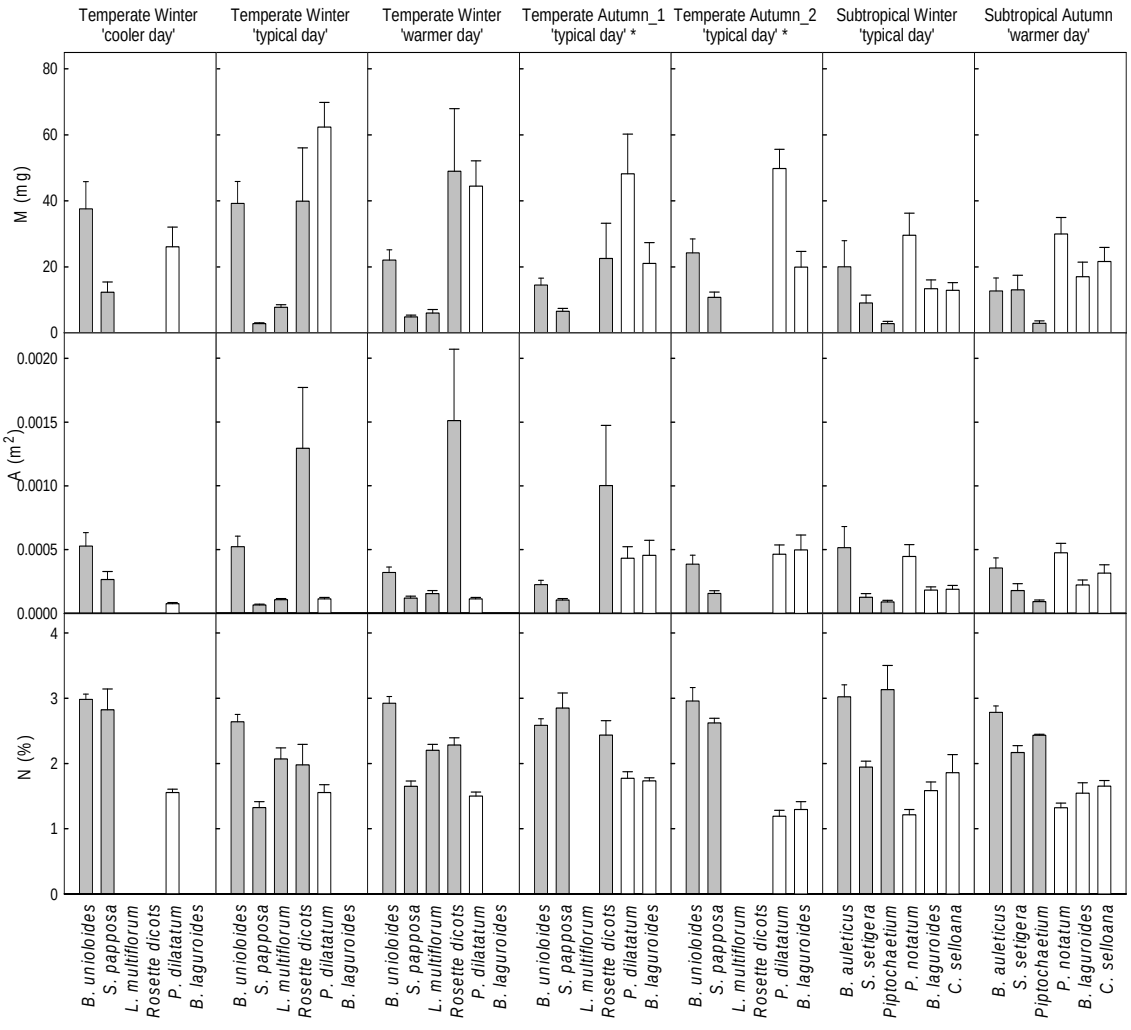

Fig. III.7. Means of carbon shoot mass (M, top panel), total blade area per shoot (A, centre panel) and nitrogen shoot percent (N, bottom panel) of C3 (grey) and C4 (white) species. Bars indicate SEM. * Temperate autumn_1 and Temperate autumn_2 refer to Exps. 11 and 12, respectively.

At the subtropical site, RPR was also lower in winter than autumn for *P. notatum* (C4), *C. selloana* (C4) and *S. setigera* (C3), but no seasonal difference was observed for *B. laguroides* (C4) and *B. auleticus* (C3), while the RPR of *Piptochaetium* sp. (C3) was lower in autumn than in winter (Fig. III.8, top panel). Opposite to the pattern observed at the temperate site, the seasonal change in RPR at the subtropical site was driven by changes in GAR in both C3 and C4 species (Fig. III.8).

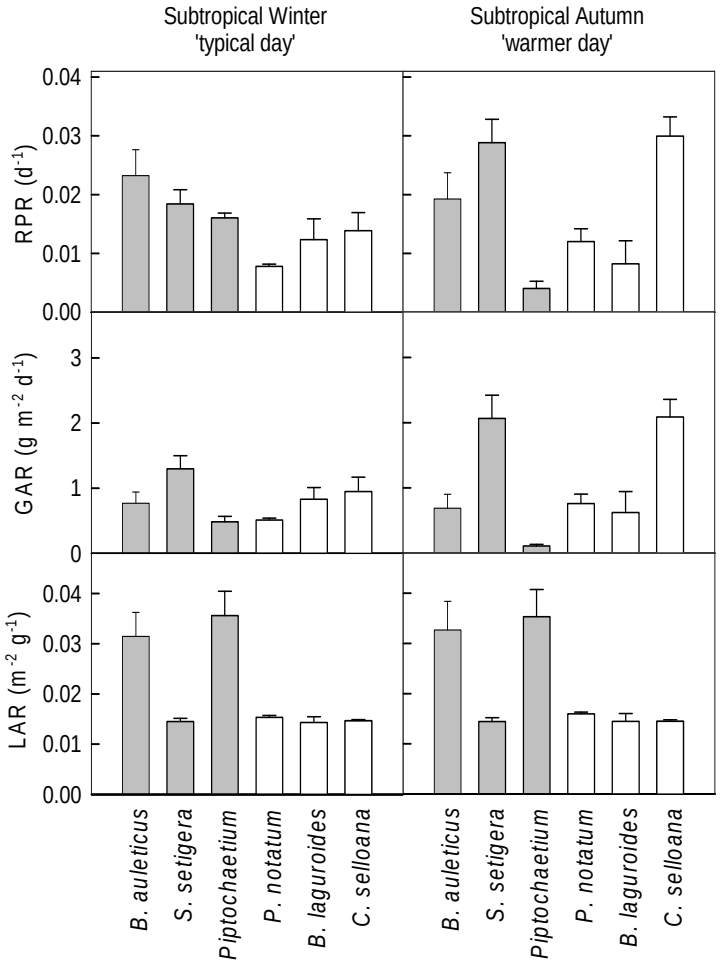

Fig. III.8. Relative photosynthesis rate (RPR, top panel), gross assimilation rate (GAR, centre panel) and leaf area ratio (LAR, bottom panel) of C3 (grey) and C4 (white) species growing at the subtropical site of Río de la Plata grasslands. Bars indicate SEM.

III.3.3 The response of individual C3 and C4 species

In the coldest environment evaluated, *i.e.* in winter at the temperate site, the lower RPR of C4 than C3 species was due to a consistently lower LAR (Fig. III.6). Differences in GAR between C3 and C4 species were less consistent and species related: *e.g.* GAR of *P. dilatatum* (C4) was similar to that of *S. papposa* and of rosette dicots, but lower than that of *B. unioloides* and *L. multiflorum* (Fig. III.6).

At milder temperatures, the component explaining the lower RPR of C4 plants was species-dependent. Thus, in autumn at the temperate site, the lower RPR of *P. dilatatum* (C4) was due to both lower GAR and LAR when compared with *S. papposa*

and *B. unioloides*, but only due to a lower LAR when compared with rosette dicots. In turn, the lower RPR of *B. laguroides* (C4) was due to a lower GAR and LAR compared with rosette dicots, but largely due to a lower GAR when compared with *S. papposa* and *B. unioloides* (Fig. III.6).

In winter, at the subtropical site, differences between C3 and C4 plants were species-dependent. All C3 species showed a higher RPR than *P. notatum* (C4), with these differences mainly explained by a lower LAR when compared with *B. auleticus* and *Piptochaetium* sp., and to a lower GAR when compared with *S. setigera* (Fig. III.8). In turn, *B. laguroides* (C4) and *C. selloana* (C4) had a lower RPR than *B. auleticus* mainly due to its lower LAR. However, *B. laguroides* and *C. selloana* showed a similar RPR as *S. setigera* and *Piptochaetium* sp. For both C4 species, the similar RPR was due to a similar LAR and GAR compared with *S. setigera* but due to a lower LAR and higher GAR compared with *Piptochaetium* sp. (Fig. III.8).

In the warmest environment evaluated, the autumn at the subtropical site, differences in RPR were species-related. *Stipa setigera* (C3) and *C. selloana* (C4) showed higher RPR due to a superior GAR (Fig. III.8). In turn, *B. auleticus* (C3) showed an intermediate RPR, which was higher than that observed in *P. notatum* (C4) and *B. laguroides* (C4). The higher RPR of *B. auleticus* was due to a higher LAR, (Fig. III.8). *Piptochaetium* sp. (C3) showed the lowest RPR of all species and this was due to its lower GAR.

III.3.4 Site effects: subtropical vs. temperate grasslands

The site effect was analyzed by comparing growing seasons with similar temperature of around 23 °C, *i.e.* the winter at the subtropical site *vs*. the autumn at the temperate site (Table III.3). In addition, the analysis was restricted to four congeneric species,

two C3s (*Bromus* and *Stipa*) and two C4s (*Paspalum* and *Bothriochloa*). Except for *Stipa*, all plants growing at the subtropical site showed higher RPR than plants growing at the temperate site. Thus, *Paspalum*, *Bromus* and *Bothriochloa* growing in winter at the subtropical site showed a higher RPR than in autumn at the temperate site (Fig. III.9). Differences between sites were mainly explained by LAR in the case of *Bromus* and *Paspalum*, and by GAR in the case of *Bothriochloa* (Fig. III.9). The similar RPR observed between sites in *Stipa* was explained by similarities in both LAR and GAR (Fig. III.9).

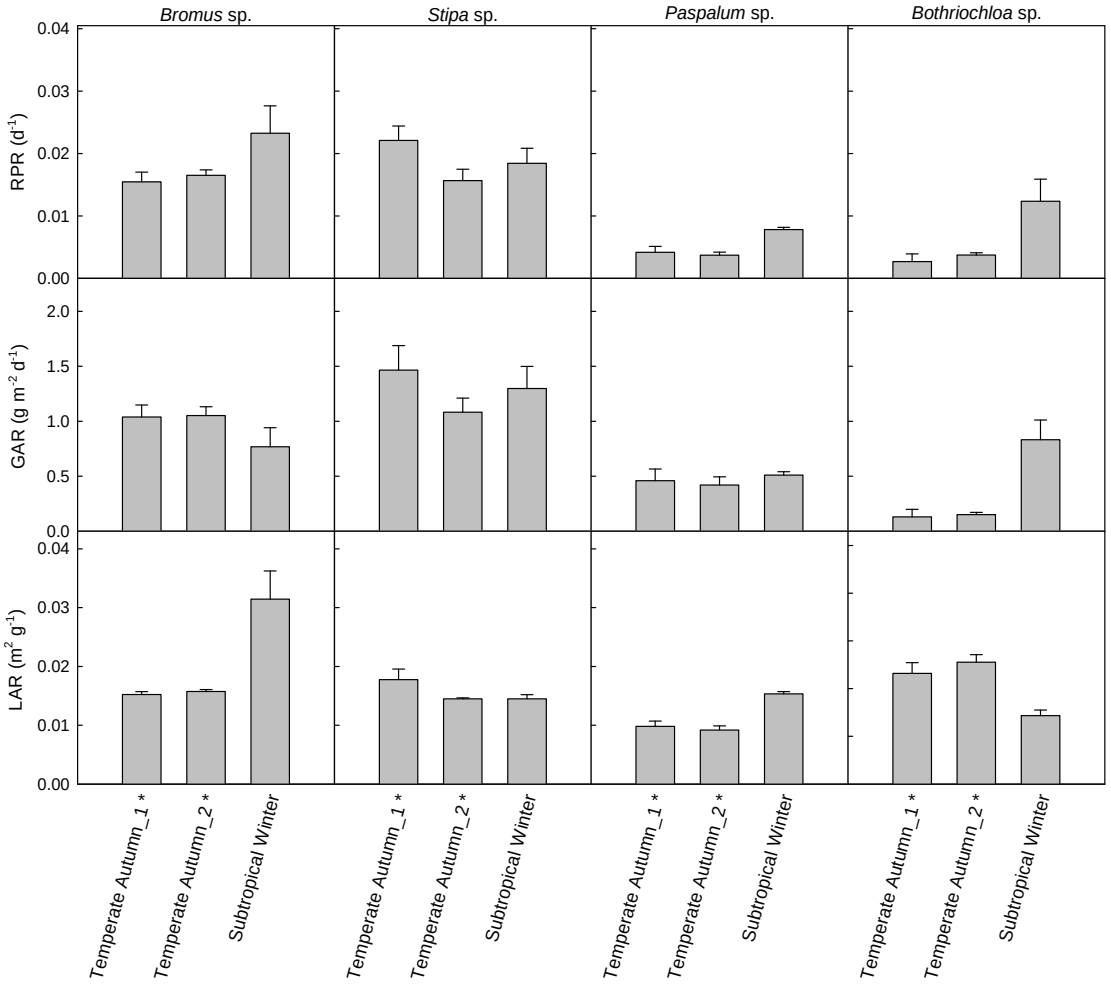

Fig. III.9. Relative photosynthesis rate (RPR, top panel), gross assimilation rate (GAR, centre panel) and leaf area ratio (LAR, bottom panel) of *Bromus* sp., *Stipa* sp., *Paspalum* sp. and *Bothriochloa* sp. growing at autumn of temperate site (Exps. 11 and 12) and winter of subtropical site (Exp. 14). * Temperate autumn_1 and Temperate autumn_2 refer to Exps. 11 and 12, respectively. Bars indicate SEM.

IV. DISCUSSION

The same three parts established in the Results chapter are followed in this section: the accuracy, strengths and weaknesses of the estimated daily carbon gain are discussed first (section IV.1); the performance of individuals and functional groups coexisting in dense, structured canopies is discussed second (section IV.2); and the role of photosynthetic capacity and leaf area ratio on the carbon gain of C3 and C4 species during the cool-season of Río de la Plata grasslands is discussed third (section IV.3).

IV.1 A new method to assess the daily carbon gain of plants in field stands

IV.1.1 On the accuracy of the estimated daily gross carbon gain

The agreement between the measured variable –^{13}C content of shoots– and the inferred variable –daily gross carbon gain of individuals– is based on the condition that no tracer is lost from shoots during the labeling period. This condition was not met in a strict sense: tracer can move belowground or be respired within hours of its assimilation. Thus, carbon gain is underestimated. But for labeling periods of up to six or seven hours, carbon gain was underestimated by 10% or less. This assessment is based on several results: (i) in the present study, tracer was detected belowground only after six hours of steady state labeling (Fig. III.1); (ii) studies which fully accounted for tracer fate found 87 % of assimilated tracer still in the shoot after eight hours of steady state labeling (Lehmeier et al. 2010a), and (iii) the same picture emerged from field experiments (Table III.1). Further, (iv) low initial tracer loss was also observed in pulse-labeling studies (review by Kuzyakov and Gavrichkova 2010): on average, tracer in belowground respiration peaked 12.5 h after the pulse and less than 9 % of all tracer was actually respired over the initial six hours (Table III.2).

Why is so little tracer lost over the initial hours of steady state labeling? Two non mutually exclusive reasons are offered. First, a large fraction of the tracer is not used immediately after its assimilation, but it resides for some time in shoot metabolic and temporary storage pools (ap Rees 1980; Plaxton & Podestá 2006; Lehmeier et al. 2008, 2010a,b), which delays its loss *via* respiration or allocation belowground. Second, in the field there is a temporal delay between the diel cycles of incident PAR (driver of carbon assimilation rate), air temperature (driver of shoot respiration rate), and soil temperature (driver of belowground activity rate). Thus, the daily increase in PAR precedes the increment in air temperature, which precedes the increment in soil temperature (Fahnestock and Knapp 1994). In consequence, tracer assimilation rate would precede tracer loss rate *via* shoot respiration, which would in turn precede tracer loss rate *via* root growth and respiration.

IV.1.2 Strengths and weaknesses of the labeling approach to quantify carbon gain of individuals

One of the aims of this thesis was to present and assess a new tool to estimate the daily carbon gain of individuals growing in field stand, in mixed canopies. Previously, estimations of daily carbon gain of individuals were done via a modeling approach, by scaling-up leaf photosynthetic responses (Barnes et al. 1990, Anten and Hirose 2003). Main inputs of such an approach are the light intercepted by, and nitrogen content of, individual leaves, and their relationship with CO_2-exchange measurements. This approach provides (and depends of) a great number of information on individuals' photosynthetic response to PAR and nitrogen allowing –for instance– the quantification of photosynthetic light- and nitrogen-use efficiencies for individual leaves located at different depths with the canopy. However, that approach relies on

three major assumptions. First, eventual differences in the temperature of leaves located at different positions within the canopy, and during the course of the day, are not accounted for and thus assumed to have a negligible effect on photosynthesis. Similarly, spatial and temporal variations in leaf water status and vapor pressure deficit within the canopy are assumed unimportant. Second, the proportions of direct and diffuse radiation intercepted by leaves within the canopy are modeled, not measured. Third, photosynthetic and respiratory responses to PAR and nitrogen are assumed equal for all leaves of a given species. In consequences, intrinsic leaf age effects, for instance, are not considered. In the same way, the approach does generally not allow for a differentiation of individuals (of the same species) in different hierarchical positions in the canopy. Further, the great number of measurements required to estimate the daily carbon gain of a single individual, renders the estimation of the carbon gain of each and every individual forming a dense stand practically impossible.

Conversely, in the labeling approach –demonstrated in the present thesis– all the effects of diurnal and spatial (within canopy) variability in environmental conditions (*i.e.* temperature, vapor pressure deficit, PAR) are accounted for, as are ontological plant-effects (*i.e.* leaf ageing). For labeling periods of up to 6 h or 7 h, carbon gain would be underestimated by no more than 10%, except possibly in seedling were higher losses seem likely probably due to both a low residence time of tracer in shoots (little storage) and a great allocation belowground. Conversely, in subordinate/shaded individuals, a presumably lower residence time of tracer in shoot pools would be counterbalanced by both a lower partitioning belowground (Minchin et al. 1994; Poorter and Nagel 2000) and the fact that respiration is dominated by the maintenance component, which seems to rely primarily on old, unlabeled carbon

(Lötscher et al. 2004).

Most important, estimating the carbon gain of all individuals, species or functional groups in a stand becomes as simple as harvesting all present individuals, species or functional groups. The labeling approach can not provide detailed information as the carbon gain of different parts of an individual plants (*i.e.* leaves at different positions within the canopy). But measurements of light capture would be complementary, enhancing the understanding of mechanisms determining the carbon gain of individuals growing in complex communities.

IV.2 Carbon gain of individuals and functional groups growing in field stands

IV.2.1 The relationship between size and carbon gain of individuals growing in dense stands

RPR correlated positively with individual's shoot mass (Fig. III.2). This was not due to increasing LAR; bigger individuals had in fact proportionally less leaf area in relation to their mass (Fig. III.4), as is often observed (Lambers and Poorter 1992). Rather, increasing GAR caused the positive relationship between RPR and individual shoot mass (Fig. III.4). This suggests that, in these crowded stands, the bigger the individual, the better its light environment, the higher the GAR, which more than offsets the lesser LAR and thus results in higher RPR.

The association between RPR and mass was nonetheless generally asymptotic (Fig. III.2). This was less evident when extended shoot height was considered, except in *P. dilatatum* (Fig. III.3). Hikosaka et al. (1999) argued that this may occur because nitrogen content per unit leaf area (N_L) starts to limit carbon gain at the high irradiances experienced by dominant individuals. N_L levels did stabilize in our study

although at high values (Fig. III.3). Another explanation is that being bigger did not translate in increased light capture (and thus no higher GAR). This occurs, for instance, when shoots adopt a more horizontal position as individuals grow bigger (Gibson et al. 1992). The fact that the extended shoot height of big individuals was greater than stand height support this interpretation (Fig. III.3 and Table II.1).

Differences in individual RPR in crowded stands are thought to reflect size-asymmetric competition for light, that is, bigger plants capture a disproportionately larger share of the resource (Weiner 1990) – a pattern verified within species but not always between species (*cf.* Hikosaka et al. 1999; Anten and Hirose 2003; van Kuijk et al. 2008) and that depends on the variable used to describe size (Berntson and Wayne 2000). Results of the present work suggest that competition within species was size-asymmetric among subordinate individuals but became size-symmetric between dominant individuals, both when mass or shoot extended height were used to describe size. In monospecific stands Ramseier and Weiner (2006) detected such a transition when individuals had bigger mass than their neighbors. In the present study, it occurred when individuals of *P. dilatatum* had higher extended shoot height than their *L. perenne* neighbors (Fig. III.3). Additional measurements of light capture and a more detailed analyses of the role of leaf length and tiller angle would help understand better these transitions.

IV.2.2 The carbon gain of functional groups: rosette dicots vs. grasses

Studies of the interaction between grasses and rosette dicots emphasize the role of stand biomass in determining the performance of rosette dicots (Russell and Spencer 2010, and references therein). More generally, in a study with a high number of wetlands species, Gaudet and Keddy (1988) argued that plant biomass predicts

competitive ability. In the present study, as observed by Barnes et al. (1990), stand dominance was a poor indicator of differences in RPR (*cf.* Fig. III.5 a-c and Fig. III.5 d-f). It was the hierarchical position of individuals groups that appeared to determine their RPR, where hierarchical position refers to the extended shoot height of an individual in relation to stand height. Indeed, contrasting allometric relationships between extended shoot height and mass (Fig. III.3) determined that interspecific differences in RPR largely disappeared when extended shoot height was considered, and hierarchically similar individuals were equally effective at capturing carbon per unit shoot mass (Fig. III.3).

Thus, in a short and dense stand (Exp. 5) rosette dicots had the same RPR than grasses. But rosette dicots had lower RPR in a taller stand (Exp. 6), and their situation worsened in a shaded stand (Exp. 7), even though stand biomass was lower in the later (Table II.1). The proposed reason is that in comparison to grasses, rosette dicots face higher costs to gain height (Fig. III.3), possibly because of the absence of a supporting structure, such as the pseudostem, and a lower length-to-width ratio of leaves. On the other hand, these characteristics would explain their ability to increase size with minimal reductions in LAR (Fig. III.4). Hence, rosette dicots are less able to put their leaves higher up in the canopy than grasses, and this has consequences for the carbon gain of this functional group under contrasting canopy structures.

IV.3 Daily carbon gain of C3 and C4 species during the autumn-to-winter transition in temperate and subtropical grasslands

IV.3.1 Leaf area per unit shoot mass limits the carbon gain of C4 individuals in cool environments

At the temperate site, a strong reduction of the LAR was observed in C4 species as the

cool-season progressed from autumn to winter. This reduction was not due to incremental selective grazing, for stocking rates were adjusted weekly to maintain a constant sward height (Agnusdei and Mazzanti 2001). Rather, it would be due to a decreased rate of leaf area production (*i.e.* lower rates of leaf appearance and/or leaf expansion), a different structure or composition of present leaves, and/or an increased rate of leaf senescence.

A much decreased rate of leaf area production seems plausible, as mean daily temperatures observed during winter were close to 8 ºC to 10 ºC, a range close to the base temperature for leaf appearance and leaf growth processes reported for several C4 species (Numata and Mitsudera 1969; Andrade 1995; Ben-Haj-Salah et al. 1995; Lemaire and Agnusdei 2000). While in *B. laguroides* the lack of over-wintering green leaves supports the idea of a complete arrest of leaf area production, plants of *P. dilatatum* had a lower number of green leaves per tiller, plus a lower blade area per leaf, in winter than in autumn. Similar results were observed when *P. dilatatum* was excluded from grazing for up to 60 days: the blade area of the last fully expanded leaf was 3 times lower in winter than in autumn (Table III.3). These results reinforce the idea that leaf area production virtually stopped in C4 plants during winter at the temperate site.

A reduced specific leaf area (SLA) may have also contributed to the lower LAR during winter of *P. dilatatum* (Cavaco et al. 2003). Lower SLA could be a consequence of a reduced cell expansion rate, leading to higher tissue density (*e.g.* content of cell wall material per unit leaf volume, Poorter et al. 2009), and/or a passive accumulation of water soluble compounds (Forde et al. 1975), as low temperatures typically decrease more the use of carbon substrates than carbon assimilation (Wilson 1966; Duncan and Hesketh 1968).

Finally, the lower LAR of C4 species during winter at the temperate site could be due to increased senescence rates, possibly associated with frost-damage consequence of the subtropical origin of some C4 species (Edwards and Still 2008). Such a mechanism seems plausible for *B. laguroides*, but no damage was observed in *P. dilatatum*'s leaves, a species native of the Río de la Plata grasslands. This confirms the reported ability of this species to sustain green leaves at low temperatures, even well below 8 °C (*e.g.* Rowley et al. 1975, Rowley 1976).

Compared to tolerant C4 species, C4 species with low tolerance to freezing show, previous to injury, lower photosynthesis rates per unit leaf area (Liu and Osborne 2008). Similar results were observed for the C4 species studied here: prior to the occurrence of frosts, GAR was higher in *P. dilatatum* (freezing tolerant) than in *B. laguroides* (presumably, non-freezing tolerant). Further, *B. laguroides* showed a higher SLA, suggesting a reduced accumulation of solutes during autumn. These results agree with the idea posed by Liu and Osborne (2008) for grasses of the semi-arid Mongolian *steppe* that frost- and drought-tolerance mechanisms (such as solutes accumulation, and higher carbon fixation) are linked in C4 species. The presence of a similar link for C4 species growing in the more humid Río de la Plata grassland deserves further research.

Interestingly, both *P. dilatatum* and *B. laguroides* are perennials, and thus able to fully regrow during the warm–season. Clearly, *P. dilatatum* and *B. laguroides* appear to have interspecific differences in their strategy to surmount the colder part of the year. While *P. dilatatum* would tolerate low winter temperatures by maintaining carbon fixation at similar levels than autumn, although with reduced leaf area production, *B. laguroides* would avoid winter as a quiescent species.

The analysis of the performance of congeneric species growing at similar PAR

and temperatures in the subtropical *vs.* the temperate site supports the idea that *Bothriochloa* and *Paspalum* differ in their strategy to deal with winter conditions. The RPR of *B. laguroides* was lower during autumn at the temperate site (~17 ºC of daily mean) than during winter at the subtropical site (~14 ºC of daily mean), mainly due to a lower GAR. This could reflect a programmed disassembling of the photosynthetic machinery, which would not have happened in plants growing at the subtropical site. Conversely, the lower RPR of *P. dilatatum* during autumn at the temperate site relative to that of *P. notatum* during winter at the subtropical site was due to a lower LAR, with no major difference in GAR.

IV.3.2 The carbon gain of C4 individuals in cool environments was not limited by leaf photosynthesis

The low RPR of C4 species at the temperate site was not related to their photosynthetic metabolism: *P. dilatatum* tillers had the same GAR during winter than during autumn. Two non mutually exclusive explanations of that seasonal stability of GAR are offered. First, in autumn, GAR may have been limited by the nitrogen content per unit leaf area. Tiller mass and nitrogen content were similar between seasons, but blade area per tiller was strongly reduced in winter (Fig. III.7). As a consequence, nitrogen content per unit leaf area of *P. dilatatum* should have been higher in winter than autumn. This could be the passive consequence of the death of older leaves, poorer in nitrogen. Indeed, the low number of green leaves per tiller observed during winter was due to the lack of old leaves (Table III.3). Young leaves have shown a similar photosynthetic-response to temperature than old ones (*i.e.* the slope of photosynthesis-temperature relationship is the same), but they have higher absolute photosynthesis rates (*i.e*. the intercept is greater), partly because of a higher nitrogen content (Woledge 1971; 1972; Robson and Deacon 1978; Woledge and

Parsons 1986; Anten et al. 1995).

Second, *P. dilatatum* leaves may have cold-acclimated, including adjusting its content and allocation of nitrogen between different photosynthetic components (Cavaco et al. 2003; Dwyer et al. 2007). Sage and Kubien (2007) state that in C4 species from cool climates the major effect of cold acclimation is a higher photosynthesis rate at moderately high temperatures (> 20 °C). Individual leaves of cold-acclimated *P. dilatatum* (grown at 10/8 ºC, day/night) did show similar photosynthetic rates at 15 ºC than non-acclimated plants (grown at 25/18 ºC, day/night) at 20 – 25 ºC (Cavaco et al. 2003). Measurements of seasonal responses of GAR, at leaf and tiller level, under different levels of nitrogen addition and including tillers with older leaves artificially excised should aid to test the relative importance of these proposed hypotheses.

Interestingly, as leaf area production was strongly reduced by low temperatures in C4 species, but the same GAR was observed in autumn and winter, low C4 performance at low temperature can not be ascribed to limited carbon supply. In fact, the main sink of carbon fixed during winter would be carbon stores (*i.e.* starch), maintenance respiration, or belowground allocation.

IV.3.3 RPR responses of the C3 and C4 functional groups during the autumn-to-winter transition

To discuss the main findings about the comparative performance of C3 and C4 species during the autumn-to winter transition, the data from Exps. 8 to 14 were rearranged in the Figure IV.1. This figure summarizes data from 235 individuals of 12 C3 and C4 species, obtained in a subtropical and a temperate grasslands during seven labeling experiments. RPR and LAR are measured quantities. GAR was derived as the ratio of RPR to LAR, and its error estimated by Gaussian propagation.

The data for each functional group in Figure IV.1 derives from averaging the means of C3 and C4 species, at each site and each season. Thus, individual species have equal weight in the mean. The species were chosen because of their dominance in the sampled canopies. At each site and season, the 4 to 6 sampled species represented more than three-quarters of the standing biomass. The annual C3 *L. multiflorum* was only present in significant amounts during winter. Rosette dicots were only present in a significant amounts at the temperate site.

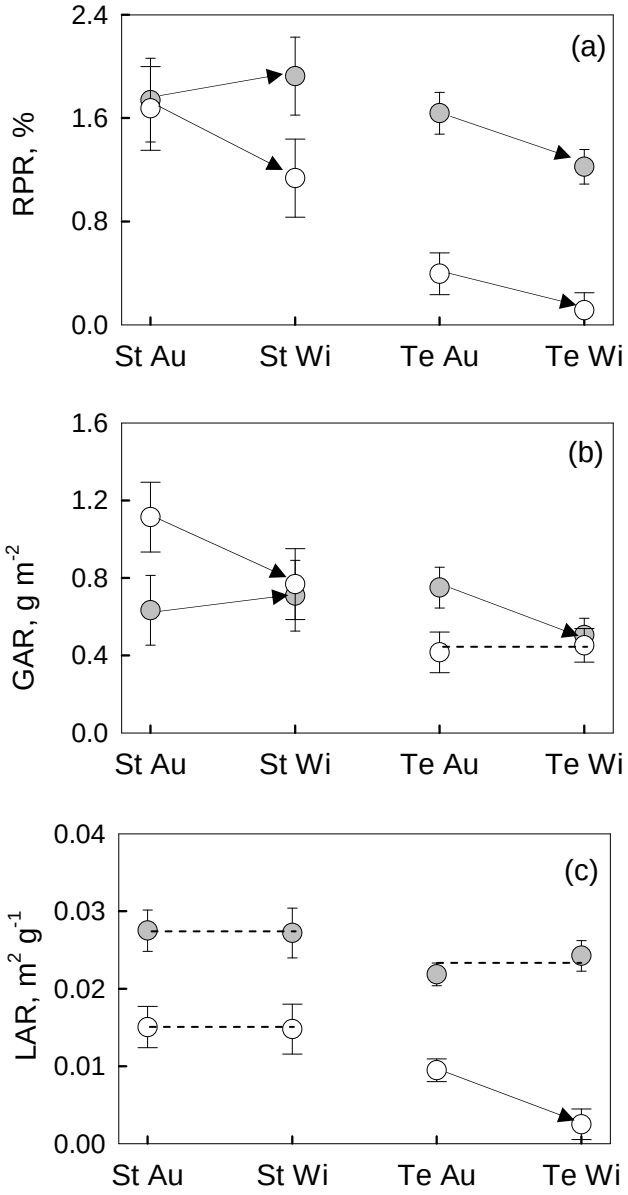

Fig. IV.1. Relative photosynthesis rate (a), gross assimilation rate (b) and leaf ratio (c) of C3 (grey) and C4 (white) species growing during autumn and winter in a temperate and subtropical site of Río de la Plata grasslands. Bars indicate the 95%-confidence interval. Solid arrows indicates that changes in a variable (*i.e.* GAR-LAR) are causes of changes in RPR. Dashed lines show no change in the variable.

Not all species of a given functional group showed the exact same magnitude of response in all variables (as previously described and discussed). However, the responses of C3 *vs.* C4 grasses generally contrasted, with only partial overlap in their behavior. It is therefore possible to talk with confidence about C3 *vs.* C4 response-

patterns. Rosettes dicots (C3) showed similar trends as C3 grasses, but in this case absolute values differed clearly: rosettes dicots had lower GAR and higher LAR than C3 grasses. In this case, because of the particularly prostrate growth habit of rosette dicots, shading can not be excluded when interpreting RPR, GAR and LAR results (see Chapters III.2 and IV.2).

In both the subtropical and the temperate site, the RPR of C3 and C4 species decreased as the cool-season progressed from autumn to winter. This reduction was relatively greater in C4 species. To my knowledge, such direct measurements of C4 performance in natural grasslands have not been done before. While novel, this result was expected, and it is associated with the well documented reduction of the contribution of C4 vegetation to aboveground biomass during the autumn-to-winter transition observed in C3/C4 mixed grasslands worldwide (Ode et al. 1980; Sage et al. 1999). Still, it is remarkable that a one-day carbon gain measurement was able to predict well the direction of change of the C3/C4 balance in the sward (Conolly et al. 2001).

Remarkably, the mechanism behind the seasonal reduction in RPR differed between sites and functional groups. In both sites, seasonal changes in the RPR of C3 species were associated with seasonal changes in GAR, that is, the capacity to fix carbon per unit leaf area. There was no change in LAR (Fig. IV.1 a-c). This same pattern was observed in C4 species at the subtropical site (*i.e.* RPR followed GAR, without changes in LAR). However, at the temperate site, the low RPR of C4 species was associated with a low LAR, as the change in GAR was minimal (Fig. IV.1 a-c). Thus, these results reveal that the failure of the most common C4 species in a cool environment was not related to their photosynthetic metabolism but was rather limited by their shoot morphology.

The current ecophysiological knowledge on C3/C4 interactions, as framed in the quantum yield hypothesis (Ehleringer 1978, 2005, Collatz et al 1998), predicts that at current atmospheric pCO_2, C3 plants should outperform C4 vegetation in environments with daytime temperatures below the 21 ºC – 26 ºC range, while C4 plants should outperform C3s at higher daytime temperatures (Ehleringer et al. 1997). In the present work, C3 species did show higher RPR than C4 plants at daytime temperatures below 20 ºC. But at daytime temperatures of 22 °C to 24°C (autumn at the temperate site, winter at the subtropical site), at which C3 and C4 species should have had a similar carbon gain, the RPR of C3 species was consistently higher than that of C4 species. Further, at daytime temperatures of 30°C (autumn at the subtropical site), where C4 species should have had a better performance than C3s, similar RPRs were observed in species of both photosynthetic pathways. Thus, the quantum yield hypothesis would appear to be only weakly supported by these data.

It is important to note that the differences in leaf photosynthesis on which the quantum yield hypothesis is based did, to some extent, appear. Indeed, GAR responses in the subtropical site followed the pattern predicted by quantum yield hypothesis, being clearly higher in the C4s at 30 °C, and similar between C3 and C4 species at 23 °C. However, at the temperate site, the GAR of C4 species was lower than that of C3 species in autumn (23 °C), but rather similar in winter (8 to 24 °C). Thus, while leaf-level carbon gain responses were partially well predicted by the quantum yield hypothesis, carbon gain of individuals –and therefore, the competitive ability of the functional group– was determined to a large extent by shoot morphology, the allocation of carbon to leaf area in particular.

In consequence, these results demonstrate the important role of the amount of leaf area per unit shoot mass to explain the performance of C3/C4 species in grazed

temperate grasslands. This suggests that differences between C3 and C4 species in their capacity to use carbon substrates in leaf area production during the cool-season may be as important as the ability to assimilate carbon in determining the expansion of C4 species into temperate-cool environments. Certainly, it would be worth to explore for intrinsic differences in the response to temperature of cell production and expansion in C3 *vs*. C4 species.

V. CONCLUSIONS

In the first part of this thesis, a ^{13}C-labeling approach to estimate *in situ* daily gross carbon gain of individuals in field stands was presented. Major advances of this technique are its mobility, simplicity, and ability to simultaneously determine the carbon gain of each individual, species and functional group present in a stand. It can be of help to physiological ecologists attempting to understand community phenomena in terms of the behavior of their components, particularly in analyses of the mechanisms of plant competition in natural stands. Measurements of light capture per individual, though not essential for the measurement of RPR, would be complementary to labeling studies and enhance the understanding of acting mechanisms.

In the second part of this thesis, the general aptitude of the new approach to test carbon gain-hypothesis was evaluated in the field. A generalized asymptotic behavior of RPR with respect to individual's mass was observed, suggesting a change from size-asymmetric to size-symmetric competition in small *vs.* large individuals. Further, a disparity between dominance and RPR of functional groups coexisting in a stand was found. Assuming differences in RPR lead to differences in relative growth rates (Hikosaka et al. 1999), these results reveal a contrast between which functional group dominated a stand and which one was gaining (*i.e.* increasing its contribution). Differentiating these two aspects of competition is important but technically challenging (Connolly et al. 2001). The possibility of a direct measure of RPR of individuals, species or functional groups –as shown here– can thus provide insight in field studies of plant competition.

Finally, the third part of this thesis focused on the daily carbon gain of C3 and C4 individuals growing in natural grasslands during the cool-season – particularly, on

the relative relevance of morphological and physiological traits in determining the daily carbon gain of individuals growing in well-lit canopies. The results confirmed a low daily carbon gain of C4 plants during the cool-season under natural field conditions. But differential mechanisms were observed between subtropical and temperate sites. At the temperate site, the daily carbon gain of C4 plants was limited by the amount of leaf area available to fix carbon, with no seasonal change in leaf photosynthetic capacity. Conversely, at the subtropical site, the daily carbon gain of C4 plants was limited by the photosynthetic capacity of their leaves, with no seasonal changes in LAR.

A major finding of this thesis was to demonstrate the relevance of morphology to the performance of C4 species at temperatures considered a barrier for C4 expansion (~ 8 ºC). Indeed, the results suggest that research efforts should not overlook the temperature responses of morphogenetic processes (*e.g.* cell production and expansion) involved in fluxes of leaf tissue (*i.e.* leaf appearance, growth and senescence) if a more complete understanding of the C3/C4 balance in cool climates is to be realized.

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APPENDIX

Classification of the sampled species.

C4 grasses

Biochemical subtype	NADP-malic enzyme (NADP-ME)		
Order	*Poales*		
Family	*Poaceae*		
Sub-family	*Panicoideae*		
Tribe	*Paniceae*	*Andropogoneae*	
Genus	*Paspalum*	*Bothriochloa*	*Coelorachis*
Species	*P. dilatatum*; *P. notatum*	*B. laguroides*	*C. selloana*

C3 grasses

Order	*Poales*				
Family	*Poaceae*				
Sub-family	*Pooideae*			*Stipoideae*	
Tribe	*Poeae*		*Bromeae*	*Stipeae*	
Genus	*Lolium*	*Poa*	*Bromus*	*Stipa*	*Piptochaetium*
Species	*L. multiflorum*	*P. pratensis*	*B. unioloides; B. auleticus*	*S. papposa; S. setigera*	*P. montevidense; P. stipoides*

C3 rosette dicot

Order	*Asterales*
Family	*Asteraceae*
Sub-family	*Cichorioideae*
Tribe	*Cichorioideae*
Genus	*Taraxacum*
Species	*T. officinalis*

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Lattanzi F.A.*, **Berone G.D.***, Feneis W. & Schnyder H. ^{13}C-labeling shows the effect of hierarchy on the carbon gain of individuals and functional groups in dense field stands. *Ecology*, In press. (*co-first authors).

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