Diss. ETH No. 17237

Determinants of tannin concentrations in forage plants. Agronomic potential of tanniferous forage plants.

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZURICH

for the degree of
DOCTOR OF SCIENCES

presented by
DIETER ADRIAN HÄRING
Dipl. Bio I (University of Basel)
born 15th of September 1976
citizen of Arisdorf, BL

accepted on the recommendation of
Prof. Dr. N. Amrhein, examiner
PD Dr. A. Lüscher, co-examiner
Prof. Dr. P. J. Edwards, co-examiner

2007

This thesis is dedicated to my wife Monika, to my parents Madeleine and Werner, my brother Reto and my sister Anita.

True knowledge is knowledge of why things are as they are, and not merely what they are.

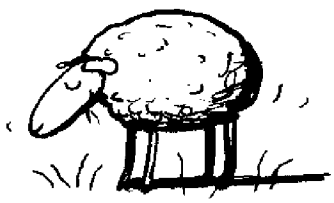
Isaiah Berlin

Change is first denied,
then vehemently opposed
and finally accepted as
being selfevident.

Bill Crabtree

All things bright and beautiful,
All creatures great and small,
All things wise and wonderful:
The Lord God made them all.

Cecil F. Alexander



Contents

List of abbreviations

ANOVA Analysis of variance

BAN Banyuls

BSA Bovine serum albumin

BuOH Butan-1-ol

CH Cinnamate hydroxylase

CHI Chalcone isomerase

CHS Chalcone syntase

CL 4-Coumaroyl-CoA ligase

CT Condensed tannins

DFR Dehydroflavonol reductase

DM Dry matter

F3H Flavonoid-3-hydroxylase

GIN Gastrointestinal nematodes

HCL Hydrochloric acid

KT Kondensierte Tannine

LAR Leucoanthocyanidin reductase

MALDI-TOF Matrix-assisted laser desorption/ionization time-of-flight (mass spectroscopy)

NMR Nuclear magnetic resonance (spectroscopy)

PAL Phenylalanine-ammonia Lyase

RUBISCO Ribulose-1,5-bis-phosphate carboxylase

SDS Sodium dodecyl sulfate

TS Trockensubstanz

Zusammenfassung

Kondensierte Tannine (KT) spielen eine Hauptrolle bei der pflanzlichen Verteidigung gegen Herbivore und Pathogene. Sie haben ein hohes Anwendungspotenzial in Nahrungsmitteln, in der Tierernährung sowie in der Human- und Tiermedizin. Kondensierte Tannine sind nicht besonders giftig, sondern sie beeinträchtigen ihre Konsumenten in einer passiven, konzentrationsabhängigen Weise indem sie die Schmackhaftigkeit und Verdaulichkeit des Pflanzenmaterials reduzieren. Diese Doktorarbeit stellt das pflanzenwissenschaftliche Modul des interdisziplinären *Tannin-Projektes* dar, welches die Erarbeitung der Grundlagen zur Nutzung von tanninhaltigen Pflanzen zur Bekämpfung von Magen-Darm-Nematoden bei Wiederkäuern zum Ziel hat. Das *Tannin-Projekt* umfasst zwei weitere Dissertationen, eine zur Wiederkäuerernährung und eine zur Parasitologie. Ziele der Doktorarbeit waren die folgenden:

1. Die Gewinnung von grundlegenden Einsichten zu wichtigen Bestimmungsgrössen der Tanninkonzentration tanninhaltiger, krautiger Pflanzen (Nährstoffverfügbarkeit, Induktion durch Elizitoren, Wachstumsgeschwindigkeit, Konkurrenz, etc.) und das Testen von bereits bestehenden Pflanzenverteidigungs-Hypothesen zur Vorhersage der Tanninkonzentration in Pflanzen.

2. Die Erforschung der agronomischen Eignung verschiedener tanninhaltiger Futterpflanzen für den Anbau und die Verwendung gegen Magen-Darm-Parasiten bei Wiederkäuern.

Das Eröffnungskapitel gibt eine kurze Einführung in die Thematik der kondensierten Tannine. Es enthält Informationen zur Biosynthese und zum Vorkommen von kondensierten Tanninen und einen Abriss über die anhaltenden Schwierigkeiten einer zuverlässigen Vorhersage der Tanninkonzentration in (wachsendem) Pflanzenmaterial. Das zweite Kapitel geht die Frage an, ob die Tanninkonzentration von *Onobrychis viciifolia* (dt. Saat-Esparsette) durch simulierte Angriffe von natürlichen 'Pflanzenfeinden' erhöht werden kann und, falls dem so ist, ob das Ausmass dieser Erhöhung vom

Nährstoff-Status der Pflanze abhängt: Die Tanninkonzentration von *Onobrychis viciifolia* nahm unabhängig vom Nährstoffangebot durch mittels Elizitoren simulierte Angriffe von Pilzen (16 %), Bakterien (20 %) oder pflanzenfressenden Insekten (29 %) in der unmittelbaren Umgebung der Verwundung zu. Trotz einer Abnahme der Tanninkonzentration mit zunehmendem Nährstoffangebot fanden sich keine Hinweise auf einen Trade-off zwischen pflanzlichem Wachstum und chemischer Verteidigung. Das dritte Kapitel behandelt die Tanninkonzentrationen verschiedener Pflanzenorgane und die Wichtigkeit der Verschiebung der Biomasseallokation in diese Organe für die Bestimmung der Tanninkonzentration auf dem Niveau der erntbaren, oberirdischen Biomasse tanninhaltiger Futterpflanzen: Sowohl in *Onobrychis viciifolia* als auch *Lotus corniculatus* (dt. Hornklee) kamen kondensierte Tannine in höherer Konzentration in Blättern (74.8, 42.6 mg KT g^{-1} TS) als in Stängeln (23.8 and 12.4 mg KT g^{-1} DM) vor. Mit fortschreitender Ontogenese (vom Säen im Mai bis zur Blattseneszenz im Oktober) stieg die Tanninkonzentration der Blätter an – gleichzeitig nahm aber der relative Anteil der (tanninreichen) Blätter an der Ernte ab (in *Onobrychis* von 100 zu 79 % DM, in *Lotus* von 61 auf 32 % DM). Anhand eines Modelles wird gezeigt, wie Wissen über die Verteilung von kondensiertem Tannin in der Pflanze und Verschiebungen der Biomasseallokation während der pflanzlichen Entwicklung für die Vorhersage der Tanninkonzentration verwendet werden kann.

Das vierte Kapitel verbindet die Arbeiten aller drei Module des *Tannin-Projektes*. Es umfasst: (i) Eine Untersuchung der agronomischen Güte von 12 Zuchtsorten vierer tanninhaltiger Futterpflanzenarten, die entweder in Reinsaat oder in Mischung mit *Festuca pratensis* (dt. Wiesenschwingel) angebaut worden waren. (ii) Eine Abschätzung der Schmackhaftigkeit von tanninhaltigen Futterpflanzen. (iii) Ein Experiment betreffend deren Wirksamkeit gegen Magen-Darm-Würmer in Schafen. Die Tanninkonzentration und die Eignung zur Kultivierung war vor allem bei *Onobrychis viciifolia* und *Lotus corniculatus* vielversprechend, während sich *Lotus pedunculatus* (dt. Sumpf-Schotenklee) unter 'normalen' Anbaubedingungen als schwacher Konkurrent zeigte und *Cichorium intybus* (dt. Wegwarte) nur sehr tiefe Tanninkonzentrationen aufwies ($<$ 10 g KT kg^{-1} TS). Die Tanninkonzentration der Ernte war unter Feldbedingungen ausgeprägten Schwankungen unterworfen, die stark vom Anteil der tanninhaltigen Pflanzen am totalen Trockenmassenertrag der Parzelle abhängig waren. Mischungen von *O. viciifolia* oder *L. corniculatus* mit *F. pratensis* waren den Reinsaaten der tanninhaltigen Pflanzen bezüglich Ertrag (Mischungen: 16.4 – 18.4 t TS ha^{-1} y^{-1} gegenüber Reinsaaten: 9.9 – 13 t TS ha^{-1} y^{-1}) und Unkrautunterdrückung überlegen. Allerdings reduzierte das Vorkommen des (nicht tanninhaltigen) Grases die Tanninkonzentration der Ernte

beträchtlich. Die Schmackhaftigkeit von getrocknetem oder siliertem *Onobrychis* oder *Lotus* war in allen Fällen zumindest vergleichbar mit einer gleichartig konservierten Gras / Klee-Mischung und, im Falle der *Onobrychis*-Silage, der entsprechenden Kontrolle sogar überlegen. Das Fressen von *Onobrychis* Heu oder Silage war mit einer Abnahme der Ei-Ausscheidung von *Haemonchus contortus*, einem der weltweit wichtigsten Schafparasiten, verbunden.

Kapitel fünf und sechs sind das Resultat einer Zusammenarbeit des pflanzenwissenschaftlichen und des parasitologischen Moduls des *Tannin-Projektes*. Sie untersuchen die parasitologische Wirkung von frischem resp. konserviertem tanninhaltigen Futter gegen Magen-Darm-Würmer in Schafen. Das Verfüttern von *Onobrychis* oder *Lotus* im Vergleich zu einem nicht-tanninhaltigen Kontrollfutter senkte den täglichen Parasiten-Ei-Ausstoss der Schafe in beiden Fällen nachhaltig um 63 % und die Anzahl der adulten Würmer um 49 resp. 35 %. Die antiparasitäre Wirkung von tanninhaltigem Futter blieb auch in konserviertem Futter erhalten.

Ich schliesse aus den präsentierten Ergebnissen, dass die Tanninkonzentration der Ernte tanninhaltiger Pflanzen mit hinreichender Genauigkeit aus der Kenntnis (i) der Pflanzenart und -sorte, (ii) dem relativen Anteil der tanninhaltigen Pflanzen an der Trockenmasse der Ernte und, im Falle von (nahezu) reinen Beständen, (iii) aus dem relativen Anteil von Blättern und Stängeln in der Ernte abgeschätzt werden kann. Bezüglich Ertrag, Schmackhaftigkeit und antiparasitärer Wirkung scheinen vor allem *Onobrychis viciifolia* und *Lotus corniculatus* aussichtsreiche Kandidaten zu sein. Eine erhöhte Konkurrenzkraft der Zuchtsorten und ein verbessertes Verständnis der Wirkungsweise der entwurmenden Aktivität von kondensiertem Tannin wäre dringend wünschenswert und bietet Forschungsbedarf für zukünftige Experimente.

Summary

Condensed tannins (CT) play a major role as plant defensive compounds in plant-herbivore and plant-pathogen interactions. Condensed tannins also have high potential for applications relevant to humans in food and animal nutrition as well as in human and veterinary medicine. Rather than being acutely toxic, condensed tannins affect their consumers in a passive, concentration-dependent manner by reducing the palatability and digestibility of the plant. This thesis represents the plant scientific module of the interdisciplinary *Tannin-Project* which is aimed at establishing basic knowledge for the use of tanniferous forages against gastrointestinal nematodes in parasitised ruminants. The *Tannin-Project* includes two other PhD-theses; one on ruminant nutrition and one on parasitology. The goals of the here presented thesis were the following:

1. To gain fundamental insight on important determinants of CT-concentrations in tanniferous, herbaceous plant species (nutrient availability, induction by elicitors, growth rate, competition, etc.) and to test already existing plant defence hypothesis for the prediction of tannin concentrations in plants.

2. To evaluate the agronomic suitability of various tanniferous forage plants for cultivation and the use against gastrointestinal nematodes in ruminants.

The opening chapter contains a brief introduction to condensed tannins. It provides information on biosynthesis and occurrence of condensed tannins and an outline concerning the continued difficulty to reliably predict the concentrations of condensed tannins in (growing) plant material. The second chapter addresses the question whether or not the tannin concentrations of *Onobrychis viciifolia* (sainfoin) can be enhanced by elicitor-simulated attacks of natural plant 'enemies' and if so, whether this induction depends on the nutrient status of the plant: in *Onobrychis viciifolia* the condensed tannin concentration in close proximity to the wound increased in response to the elicitor-simulated presence of fungi (16 %), bacteria (20 %) and herbivorous insects (29 %), independent of the nutrient status of the plant. Despite a decreasing tannin concentration with increasing nutrient availability, there was no evidence for a trade-off between

growth and defense. The third chapter deals with tannin concentrations in various plant organs and the importance of shifts in biomass allocation to these organs for the determination of tannin concentrations on the level of the harvestable aboveground biomass of tanniferous forage plants: in both *Onobrychis viciifolia* and *Lotus corniculatus* (birdsfoot trefoil), condensed tannins occured in higher concentrations in leaves (74.8, 42.6 mg CT g^{-1} DM, respectively) than in stems (23.8 and 12.4 mg CT g^{-1} DM, respectively). With progressing ontogenesis (from sowing in May to leaf senescence in October), the tannin concentrations in leaves increased – however, at the same time the relative contribution of (tannin-rich) leaves to dry matter yield decreased (*Onobrychis*: from 100 to 79 % DM, *Lotus*: from 61 to 32 % DM). In a model, it is shown how knowledge of the distribution of condensed tannins within plants and of shifts in biomass allocation during ontogenesis can be used to predict tannin concentrations.

The fourth chapter integrates work of all three modules within the *Tannin-Project*. It includes: (i) An investigation of the agronomic performance of 12 cultivars of 4 tanniferous forage species, sown either as pure stands or in mixture with *Festuca pratensis*. (ii) An assessment of the palatability of tanniferous forages. (iii) An experiment addressing the efficacy of tanniferous forage plants against gastrointestinal parasites in sheep. The tannin concentrations and the suitability for cultivation were particularly promising in *Onobrychis viciifolia* and *Lotus corniculatus* while *Lotus pedunculatus* (big trefoil) proved to be a weak competitor under 'normal' agronomic conditions and *Cichorium intybus* had very low tannin concentrations ($<$ 10 g CT kg^{-1} DM). Tannin concentrations of the harvestable biomass under field conditions showed pronounced dynamics according to the relative contribution of tanniferous plants to the total dry matter yield of the entire plot. Mixtures of *Onobrychis viciifolia* and *Lotus corniculatus* with *Festuca pratensis* were superior to purely sown stands of these tanniferous species with regard to yield (mixtures: 16.4 – 18.4 t DM ha^{-1} y^{-1} *versus* purely sown stands: 9.9 – 13 t DM ha^{-1} y^{-1}) and resistance to weed invasion. However, the presence of the (non-tanniferous) grass reduced the tannin concentrations of the harvest considerably. The palatability of dried or ensiled *Onobrychis* and *Lotus* was at least comparable to an equally conserved grass / legume mixture and, in the case of ensiled *Onobrychis*, even superior to the control. The feeding of *Onobrychis* hay or silage was associated with a reduction of the faecal egg count of *Haemonchus contortus*, one of the most important sheep parasites world-wide.

Chapters five and six are the result of a cooperation between the plant scientific and the parasitological module of the *Tannin-Project* and address the anthelmintic effect of

fresh and conserved tanniferous forages, respectively, against two important gastrointestinal nematodes in sheep. The feeding of *Onobrychis viciifolia* and *Lotus corniculatus* sustainably reduced the daily faecal egg output by 63 % (both species) relative to the respective controls and tended to lower the number of adult parasite worms by 49 and 35 %, respecively. The antiparasitic effect of tanninferous forages was largely preserved in conserved forage.

Based on the results presented herein, I conclude that tannin concentrations of harvestable biomass are reasonably well predictable from (i) the identity of the tanniferous forage plant, (ii) the relative contribution of tanniferous forages to the total dry matter yield and, in case of (almost) pure stands, (iii) from knowledge of the proportion of leaves and stems in the harvest. With regard to yield, palatability and efficacy against gastrointestinal parasites, *Onobrychis viciifolia* and *Lotus corniculatus* are particularly promising candidate plants for the control of gastrointestinal parasites, though an enhanced competitive ability of the tanniferous plants is desirable and an improved mechanistic understanding of the antiparasitic effects of condensed tannins an important area for future research.

Chapter 1

General introduction

1.1 What are condensed tannins?

Condensed tannins, or synonymously termed proanthocyanidins, are polyphenolic secondary plant metabolites. They are synthesized along the flavonoid pathway and are mainly found in woody plants but also occur in some herbaceous species; for example in some representatives of the Fabaceae plant family. *Condensed tannins*, as opposed to *hydrolysable tannins*[1], consist of covalently C-C bound flavan-3-ol units. Tannins owe their name to their capacity to bind and crosslink protein and their application in the tanning process of hides. The binding to proteins and other macromolecules is probably also the most important feature in the ecological context where condensed tannins play an important role as chemical plant defences against herbivores and pathogens (Brownlee et al., 1990; Bernays, 1981). For example, the binding of condensed tannins to salivary proteins is responsible for the astringent taste of tanniferous plant parts and can protect them from being eaten. Condensed tannins have attracted interest as antibiotics and antioxidants in human medicine (Karou et al., 2005; Cos et al., 2004; Barreiros et al., 2000; Haslam, 1996). They are important food components, for example in nuts or in red wine (Mueller-Harvey, 2006; Bogs et al., 2005). In agronomy, they have traditionally been known as digestibility reducers, however, recent experiments demonstrated beneficial impacts of condensed tannins on health and productivity of

[1] Hydrolysable tannins consist of a sugar core (usually glucose) onto which gallic or ellagic acids are linked by esterification (Mueller-Harvey, 2001; Waterman & Mole, 1994). As their name implies, hydrolysable tannins can be subjected to hydrolysis under weakly acid or weakly alkaline conditions. Traditionally, hydrolysable tannins, in contrast to condensed tannins, have often been regarded as digestible and toxic. However, today's accumulated evidence is not in favour of such generalizations (Mueller-Harvey, 2006; Terrill et al., 1994; Bernays, 1981)

ruminants. Fed at moderate concentrations, condensed tannins can reduce the risk of bloat, increase the ruminant's supply of proteins and essential amino acids, and act as antiparasitic agent against gastrointestinal nematodes (Hoste et al., 2006; Mueller-Harvey, 2006; Min et al., 2003; Aerts et al., 1999; Barry & Mcnabb, 1999).

1.2 Biosynthesis of condensed tannins

Condensed tannins are synthesized as one of several branches of the flavonoid pathway which represents a segment of the phenylpropanoid pathway (Fig. 1.1 on the facing page). Along the phenylpropanoid pathway, phenylalanine (shikimate pathway) is deaminated to *trans*-cinnamate by the enzyme phenylalanine-ammonia lyase (PAL). In several enzymatically catalysed steps, *trans*-cinnamate is then transformed to 4-coumaroyl-coenzyme A. At the start of the flavonoid pathway, the enzyme chalcone synthase (CHS) sequentially condenses one molecule coumaroyl-CoA with three molecules malonyl-CoA, which is accompanied by the loss of three molecules CO_2; the acetate units form the A-ring of the flavonoid skeleton. The enzyme chalcone isomerase (CHI) catalyses the formation of the flavanone naringenin and the closure of the C-ring. Flavonoid-3-hydroxylase (F3H) then introduces the characteristic hydroxyl-group in the C-3 position (C-ring). Dihydroflavonol reductase (DFR) reduces flavanones to form leucoanthocyanidins and leucoanthocyanidin reductase (LAR) catalyses the reduction of leucoanthocyanidins to flavan-3-ols (e.g. catechin). Up to the formation of flavan-3-ols, most enzymes are soluble and do not display significant transmembrane domains (Marles et al., 2003). They are thought to be localized on the cytoplasmic face of the endoplasmatic reticulum or the vacuolar membrane. So far, no enzyme has been demonstrated to catalyse the polymerization step of condensed tannins or their translocation into the vacuole where they are usually found (Marles et al., 2003; Suzuki et al., 2003; Abrahams et al., 2003).

1.2.1 Structural diversity of condensed tannins

Condensed tannins exist as water-soluble oligomers with two to six flavan-3-ol units, and as larger, often insoluble polymers (Haslam, 1996). The flavan-3-ol units are usually interlinked between the C-4 and the C-8 position as displayed in figure 1.1 but can also form (branched) C-4 : C-6 linked chains (Marles et al., 2003; Schofield et al., 2001). In most plants, polymers are of the highest quantitative significance, but usually monomers, dimers, trimers, etc. co-occur (Waterman & Mole, 1994). In addition to

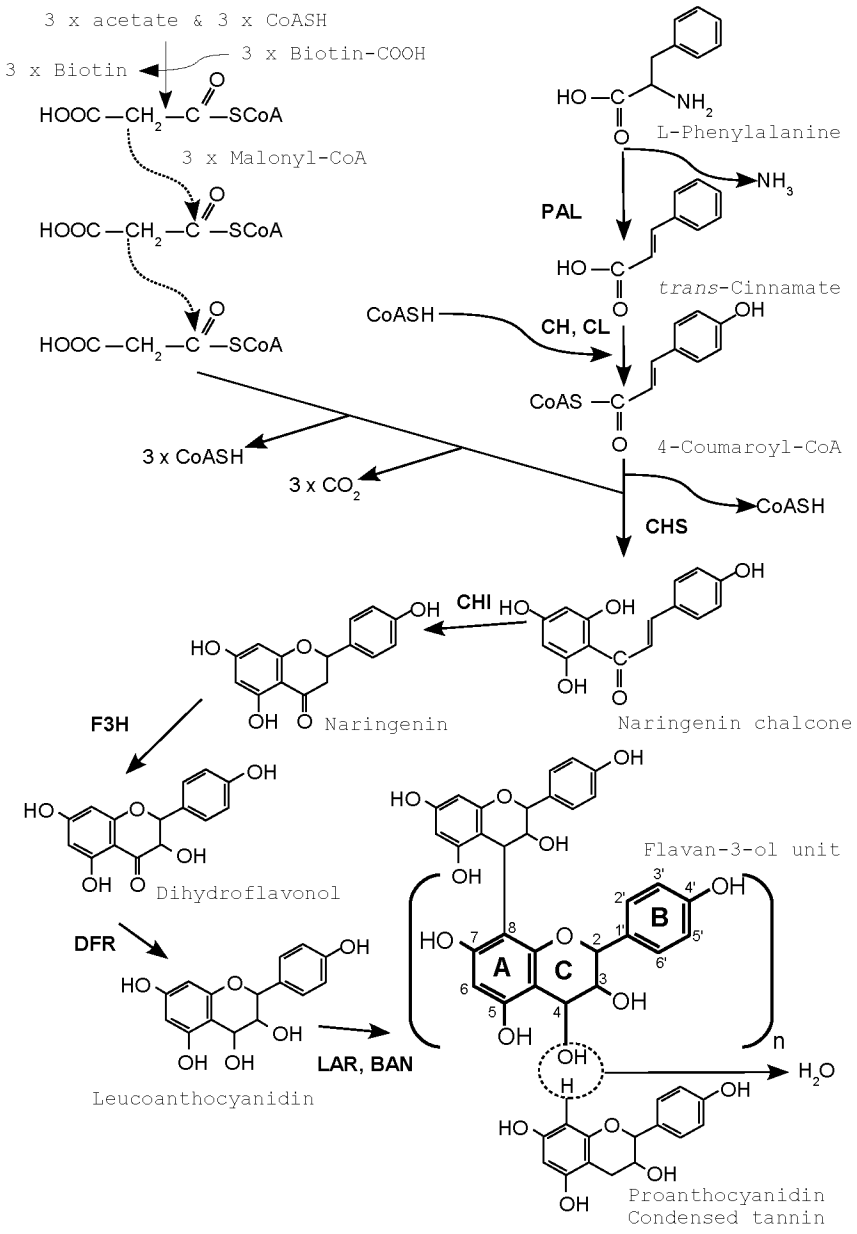


Figure 1.1: *The biosynthetic pathway leading to condensed tannins starting with the primary metabolites phenylalanine (shikimate pathway) and 3 molecules malonly coenzyme A. PAL = phenylalanine-ammonia lyase, CH = cinnamate hydroxylase, CL = 4-coumaroyl-CoA-ligase, CHS = chalcone syntase, CHI = chalcone isomerase, F3H = flavonoid-3-hydroxylase, DFR = dehydroflavonol reductase, LAR = leucoanthocyanidin reductase, BAN = Banyuls. CoASH = coenzyme A. For the creation of this diagram various sources have been considered: Marles et al. (2003); Xie et al. (2003); Waterman & Mole (1994); Mohr & Schopfer (1992).*

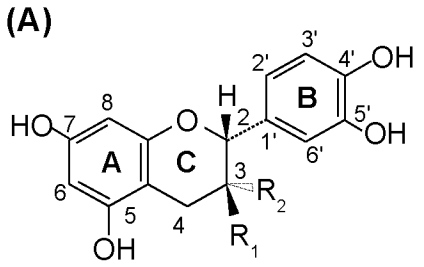
variations in size and the position of the linkage between monomers, condensed tannins can vary in structure; through the oxygenation patterns on rings A and B of the flavan-3-ol unit (e.g. C-3', C-5') and, more subtly, in the steric relationship between the aromatic substituent at C-2, the hydroxyl at C-3 and the inter-monomer bond on C-4 (e.g. catechin, epicatechin; Fig. 1.2 on the next page; Schofield et al. 2001; Haslam 1996). Additionally, substituents like gallic acid or cinnamic acids can be added to the hydroxyls, notably in the C-3 position (Waterman & Mole, 1994). Taken together, variations in the number of monomers, the stereochemistry of these monomers and the positions between which they are interlinked, the oxygenation pattern of the monomers and potential secondary addition of functional groups permit almost infinite possibilities for structural variation of condensed tannins. Moreover, the presence or absence of condensed tannins, the degree of polymerization and the diversity and identity of chemical structures varies with plant species, plant tissue and even with the developmental stage of a given plant tissue (Marles et al., 2003; Hyder et al., 2002; Singh et al., 1997; Skadhauge et al., 1997; Koupai-Abyazani et al., 1993).

1.2.2 Properties of condensed tannins

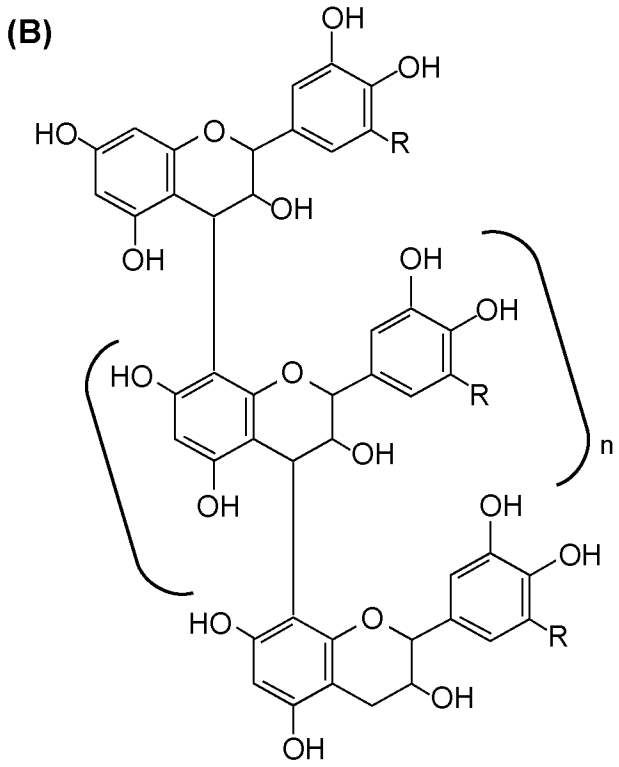
As seen above, the possibilities for structural variability of condensed tannins are almost infinite and it is inevitable that different condensed tannins differ with regard to their physical, chemical and biological properties (Mueller-Harvey, 2006). Nevertheless, condensed tannins have certain predictable properties whatever their source and, in their functionality, do not differ as widely as, for example, the alkaloids which could be as different as strychnine and caffeine (Waterman & Mole, 1994). Primarily associated with the possession of phenolic nuclei within the polymer (B-ring), important properties of condensed tannins include:

1. their complexation with metal ions,
2. their antioxidant and radical scavenging activities and
3. their ability to complex with macromolecules; notably with proteins, carbohydrates, polysaccharides and cell membranes (Haslam, 1996; Su et al., 1988).

It is widely accepted that the binding to macromolecules, in particular the complexation and crosslinking of proteins, is the most important property of condensed tannins (Mueller-Harvey, 2006; Ayres et al., 1997; Haslam, 1996; Waterman & Mole, 1994). The phenolic group of the condensed tannins is an excellent hydrogen donor that can



R1 = OH, R2 = H: Catechin
R1 = H, R2 = OH: Epichatechin



R = H: Procyanidins
R = OH: Prodelphinidins

Figure 1.2: ***A)*** *The most important monomers, catechin and epicatechin; the hydroxyl on C-3 and the B-Ring on C-2 stand on opposite sides (trans) or on the same side (cis) of the C-Ring, respectively.* ***B)*** *The procyanidins and prodelphinidin ratio (hydroxylation pattern of the B ring) may be an important determinant of the nutritive quality of condensed tannins (Mueller-Harvey, 2006; Hedqvist et al., 2000).*

form strong hydrogen bonds with the carbonyl groups of the protein backbone. Tannin-protein interactions are relatively specific and the binding strengths in different tannin-protein complexes can vary over orders of magnitude, depending on the structure of both the protein and the tannin (Hagerman & Butler, 1981; Mueller-Harvey, 2006): It is known that proteins with an open structure (α-helices or random coil) have a higher affinity to tannins than those with a compact globular structure (Hagerman & Butler, 1981). Furthermore, proteins with a high content of proline and hydroxyproline (e.g. collagen) have an especially high affinity to tannins; this is caused by the fact that the X-proline bond is a particularly strong hydrogen bond acceptor (Hagerman & Butler, 1981).

Known characteristics of condensed tannins that favor strong bonding to proteins are a high molecular weight and a high conformational mobility. The importance of the oxygenation patterns of the B ring and the conformation of the monomers are currently under investigation (Fig. 1.2 on the preceding page; Mueller-Harvey 2006). Apart from that and despite considerable efforts (e.g. Mueller-Harvey, 2006; Kraus et al., 2003b; Hedqvist et al., 2000; De Bruyne et al., 1999; Ayres et al., 1997; Clausen et al., 1990; Hagerman & Butler, 1980) we do not yet have a useful organizing principle to infer the biological function and activity from the knowledge of the chemical structure of condensed tannins (Mueller-Harvey, 2006; Waterman & Mole, 1994).

Part of the difficulty to establish general rules of the biological activity of condensed tannins is that the molecules with which (mixtures of differently structured) condensed tannins interact differ between different organisms, and are often even unknown. Therefore, it might be biologically unrealistic to expect simple and general rules for the relationship between the chemical structure of condensed tannins and its biological activity across all possible target species ('super tannins'). However, it should be possible to define rules with regard to a specific partner molecule, for example to ribulose-1,5-bisphosphate carboxylase which accounts for up to 40 % of the forage protein (Jackson et al., 1996).

As a crude and approximative measure the biological activity of condensed tannins is usually thought to be a function of the *concentration*[2] of condensed tannins defined

[2]In ecology, the term *concentration* is also used to describe the fraction between the amount of a substance of interest and the biomass of the plant tissue in which it is found. In contrast to *content*, which is used interchangeably to describe both the absolute and relative amount of a substance, *concentration* is only applied as defined in equation 1.1 on the next page of this thesis.

here as the ratio between the amount of tannins (T) and the amount of biomass (B):

$$[CT] = \frac{T}{B} \qquad (1.1)$$

1.2.3 Concerning the chemical analysis of condensed tannins

There are many different methods to dry plant samples and to extract and analyse condensed tannins – all potentially influential on the outcome of the chemical analysis and the interpretation of the results (Mueller-Harvey, 2006; Stewart et al., 2000; Schofield et al., 2001; Hedqvist et al., 2000; Waterman & Mole, 1994; Terrill et al., 1992, 1990; Hagerman, 1987). The analytical methods range from relatively inexpensive and simple to perform procedures like the radial diffusion method (Hagerman, 1987), the vanillin reaction (Price et al., 1978) or the butanol-HCL assay (proanthocyanidin method; Porter et al., 1986; Terrill et al., 1992) to equipment-intensive methods like MALDI-TOF mass spectroscopy (Behrens et al., 2003; Hedqvist et al., 2000) or NMR-spectroscopy (Thompson & Pizzi, 1995). The analytical methods differ not only with regard to cost and precision but also (and much more importantly) with regard to the feature of the tannins they address. Some methods, like the radial diffusion methods assume a priori that the bioactivity of tannins is caused by their affinity to proteins. Therefore, they quantify the interaction between condensed tannins and a specific protein (for an interesting attempt with RUBISCO see Jackson et al., 1996). Others, like the vanillin or the butanol-HCL assay, aim at a quantification of the number of monomers within a tissue. MALDI-TOF mass spectroscopy and NMR-spectroscopy can be used to assess the degree of polymerization and the nature of the flavan-3-ol monomers of the condensed tannins, respectively (Behrens et al., 2003; Thompson & Pizzi, 1995).

It was the joint decision of the people involved in the *Tannin-Project* to use the butanol-HCL assay based on Porter et al. (1986) as it is described in Terrill et al. (1992) throughout this thesis and also in the work performed by the other partners of the *Tannin-Project*. This choice has been made mainly due to the fact that most of the published work done in agronomy, animal nutrition and parasitology has been based on this approach. We therefore aimed to maximize the comparability of our results to results of others in the same field.

The method developed by Terrill et al. (1992) features the hydrochloric acid-catalysed depolymerization of the condensed tannins and, in direct proportion to the number of

monomers, yields a red anthocyanidin product that can be quantified spectrophotometrically. Terrill et al. (1992) demonstrated that even after a thorough extraction with a water / acetone solution, a relatively large fraction of condensed tannins is still bound to proteins in the plant material. Therefore, the method additionally involves a hot extraction with an SDS-mercaptoethanol-solution and the subsequent quantification of the so-called protein-bound tannins.

1.3 Occurrence of condensed tannins

It is very likely that the early evolution of land plants was intimately linked with the expansion of the phenylpropanoid pathway and the production of phenolic metabolites. Certainly, biochemical relatives of condensed tannins like cutin and lignin, and probably flavonoids were indispensable components of the rise in land plants in the Silurian and Devonian (Kubitzki, 1987). Cutin is vital to water retention and contains simple cinnamic acids, lignins are polymers of cinnamyl alcohols which are necessary for growing tall in a high-gravity environment and for the conduction of water over long distances (e.g. trees, bamboo; Waterman & Mole, 1994). It is in the pteridophytes that the formation of lignin and the production of condensed tannins started to occur. Today, the woody habit and the production of condensed tannins seem still closely linked but not completely correlated phenomena (Waterman & Mole, 1994). Exceptions to this rule are found, for example, in arboreal taxa like Oleaceae, Bignoniaceae or Violaceae which are free of condensed tannins and in condensed tannin-producing herbs belonging to the Fabaceae, Rosacea or Geraniaceae plant families (Waterman & Mole, 1994).

1.4 Importance of condensed tannins

1.4.1 Ecological relevance of condensed tannins

Condensed tannins are best known as plant defensive compounds against herbivores and pathogens (Stamp, 2003; Herms & Mattson, 1992; Edwards, 1992; Coley et al., 1985; Bryant et al., 1983; Feeny, 1976), but they have also been reported to play important roles in virtually any interaction a plant can have with other living organisms (Waterman & Mole, 1994). For example, condensed tannins can affect plant-plant interactions through a depressing effect on the degradation rate of plant litter, thereby affecting soil quality: the ecological consequences may be a reduced ecosystem pro-

ductivity and altered successional pathways (Kraus et al., 2003a).

Remembering that condensed tannins probably evolved together with the woody habit of plants, lignin and condensed tannins may originally have represented defences of dead tissues such as the heartwood of trees where further metabolism is not usually an option (Waterman & Mole, 1994; Kubitzki, 1987; Kemp & Burdon, 1986). Today, condensed tannins are commonly seen as 'a first line of chemical plant defence', meaning that they have some antibiotic properties, but in an unspecific, passive way that contrasts with the active and aggressive antibiotic activity seen in cardiac glycosides or some alkaloids (Haslam, 1996; Waterman & Mole, 1994). In this sense, condensed tannins are rarely acutely toxic but rather exert a concentration dependent plant protective effect against consumers like pathogenic fungi (Heil et al., 2002; Brownlee et al., 1990) or herbivores (Herms & Mattson, 1992; Coley et al., 1985; Bernays, 1981; Feeny, 1976).

Considering the accumulated evidence of food selection and feeding experiments, one fact emerging is the wide range of concentrations of condensed tannins and other phenolics that different consumers can tolerate (Waterman & Mole, 1994; Bernays, 1981). The typical result of food selection experiments is that herbivores avoid the consumption of levels of tannins in excess of those in their normal diet. Between consumers, the level of tolerance against condensed tannins can range from no tolerance at all to relatively high levels of tolerance and putative counter-adaptations like the production of proline-rich salivary proteins that inactivate tannins[3] (Shimada, 2006; Austin et al., 1989).

1.4.2 Applications of condensed tannins with relevance to humans

Currently, condensed tannins are being investigated in various scientific areas including food science, human and veterinary medicine and in animal nutrition. In food sciences there is a particular interest in an improved understanding of the regulative mechanisms of the synthesis of condensed tannins in grapes and the relationship between the chemical structure of the tannins and their impact on the sensory features of red wine where they act as a natural preservatives (antioxidants) but also as major contributors to the wine's flavour, texture and structure (e.g. Bogs et al., 2005; Herderich & Smith, 2005; Su et al., 1988).

[3] See section 1.2.2 on page 4.

In human medicine, condensed tannins are mainly known as antibacterial and antioxidative agents providing protection against radical-mediated injury and cardiovascular disease (Karou et al., 2005; Cos et al., 2004; Bagchi et al., 2000; Cowan, 1999; Haslam, 1996; Su et al., 1988). A review by Haslam (1996) contains a list of noteworthy pharmaceutical activities of condensed tannins including bactericidal, anthelmintic and antihepatoxic activity. Furthermore, inhibition of human immunodeficiency virus (HIV) replication, suppression of the glucosyl tranferases of *Streptococcus mutans* (dental carries) and host-mediated antitumour activity have been reported.

In agronomy, animal nutrition and veterinary medicine, it is known that condensed tannins can reduce palatability and digestibility of forages (Mueller-Harvey, 2006; Titus et al., 2000; Aerts et al., 1999; Barry & Mcnabb, 1999). However, it is also known that at low to moderate concentrations, condensed tannins can affect the performance and health of ruminants positively (Mueller-Harvey, 2006; Min et al., 2003; Aerts et al., 1999; Barry & Mcnabb, 1999). Condensed tannins have been reported to reduce the risk of bloat, to increase the ruminants' supply with essential amino acids and, thus, to increase the life weight gain, ovulation rates, and the production of milk and wool (Mueller-Harvey, 2006; Min et al., 2003; Aerts et al., 1999; Barry & Mcnabb, 1999). All of these effects are usually attributed to the ability of condensed tannins to reversibly bind to proteins under approximately pH-neutral conditions in the rumen, thereby protecting the dietary protein from premature degradation by proteolytic rumen bacteria. Further along the digestive tract, namely in the abomasum at pH < 3, these tannin-protein complexes dissociate and the protein can be digested and absorbed by the ruminant. The resulting surplus of protein is sometimes referred to as *bypass-protein* (Mueller-Harvey, 2006; Min et al., 2003). Moreover, condensed tannins have been reported to have antiparasitic effects against gastrointestinal nematodes (reviewed by Hoste et al., 2006) including a stimulation of the immunological response, a lowered parasite egg excretion and sometimes lowered worm burdens (e.g. Niezen et al., 2002).

1.5 The prediction of condensed tannin concentrations in plant material

An improved understanding of the drivers of changes of the concentration of condensed tannins in plant material is a major interest in all the different disciplines mentioned in

the previous section; be it for the practical application in food science and agronomy or for the theoretical understanding of plant-herbivore and plant-pathogen interactions in ecology. The wide-spread perception that the primary role of secondary metabolites in general, and of condensed tannins in particular, is in plant defense (Edwards, 1992) led to the formulation of a collection of ecological, evolutionary and physiological *plant defence hypotheses*, collectively known as *plant defence theory* (Stamp, 2003; Herms & Mattson, 1992; Coley et al., 1985; Feeny, 1976; McKey, 1974). These approaches were paralleled by considerable, but scientifically rather independent, efforts spent on the molecular exploration of the biosynthetic pathway of condensed tannins (reviewed by Marles et al., 2003).

1.5.1 Ecological plant defence hypotheses

Ever since the observation that many secondary plant metabolites can have a deterrent or detrimental effects on their consumers, it has been a central goal in plant-herbivore interactions to understand, explain and predict variations in the phenotypic, genotypic and geographic variation of these 'plant defences' (Stamp, 2003). With the formulation of the *optimal defence* (McKey, 1974) and the *apparency hypotheses* (Feeny, 1976) the quest for a uniforming plant defence theory started (Rhoades & Cates, 1976):

Optimal defence hypothesis

The optimal defence hypothesis states that the level of defence is predictable from (i) the *risk* of an attack, (ii) the *value* of the plant part lost and (iii) the *cost* of the defensive compound (Rhoades & Cates, 1976; McKey, 1974). For example, defence is increased when enemies are present and decreased when they are absent (Stamp, 2003). Defensive compounds should be concentrated in those regions of the plant in which their presence would maximize the fitness of the plant; outer cells layers of a plant organ, for example, should have greater chemical-defence needs than inner cells layers in the same region because they are more likely to come into contact with a potential herbivore (McKey, 1974).

Apparency hypothesis

It has been suggested that *apparent* plants – that is those with a high risk of being discovered (because they are either large or otherwise conspicuous) – make large in-

vestments in unspecific and costly[4] but broadly effective plant defences like condensed tannins (Feeny, 1976). Such defences are known as *quantitative defences* or as *digestibility reducers*. In contrast, *unapparent* plants are suggested to escape a herbivore more easily. Therefore, they usually have smaller levels of chemical defences which are already highly toxic at relatively low concentrations.

Growth-rate (or resource availability) hypothesis

Coley et al. (1985) proposed that the resource availability and intrinsic growth rates are major determinants of both the amount and type of plant defences. They suggested that when resources are limited, plants with inherently slow growth rates are favoured over those with fast growth rates; slow rates in turn, so it was proposed, favour large investments in antiherbivore defences. *The optimal level of defence investment increases as the potential* (intrinsic) *growth rate of the plant decreases given that the herbivore pressure remains constant*. This is justified as follows: (i) *as the potential growth rates become more limited by resource availability, replacement of resources lost to herbivores becomes more costly.* (ii) *A given rate of herbivory (grams of leaf removed per day) represents a larger fraction of the net production of a slow-grower than that of a fast-grower. Therefore, because the relative impact of herbivory increases as inherent growth rate declines, higher defences are expected in slower growers than in faster ones.* (iii) *A percentage reduction in growth rate due to the cost of producing defences represents a greater absolute growth reduction for fast-growing species than for slow-growing ones.*

Carbon-nutrient balance hypothesis

The carbon-nutrient balance hypothesis (CNB; Bryant et al., 1983; Bloom et al., 1985) is aimed at predicting phenotypic type and level of chemical plant defences from the relative availability of carbon and nutrients in the environment. It argues that photosynthesis is less strongly affected by nutrient shortage than plant growth. From that it deduces that carbohydrates and *carbon-based*[5] *secondary metabolites* like condensed tannins

[4]The cost of condensed tannins is assumed to be high because of the large size of the molecules and the relatively large amount of condensed tannins needed in order to produce a plant defensive effect.

[5]Carbon-based secondary metabolites is a common expression in plant defence theory to describe secondary metabolites that consist of C, O and H exclusively. In this sense terpenoids or tannins are carbon based secondary metabolites. In contrast, N-containing compounds like alkaloids are sometimes referred to as nitrogen-based secondary metabolites.

accumulate. With regard to the type of secondary metabolites, it is predicted that under nutrient limitation *carbon based secondary metabolites rise, whereas nitrogen-based defences decline*.

Growth-differentiation hypothesis

The growth-differentiation hypothesis (GDB; Herms & Mattson, 1992) predicts a trade-off between growth (i.e. cell division and enlargement) and differentiation processes (i.e. everything else including secondary metabolism). The major premise is that *the allocation of resources by plants to chemical and structural defences decreases growth by diverting resources from the production of leaf area and other vegetative structures*. It predicts that during periods of intense growth, secondary metabolism decreases because of substrate limitation. For example, *phenylalanine is the rate-limiting precursor for phenylpropanoid synthesis (e.g. lignin, flavonoids and condensed tannins), and at the same time is an essential amino acid for protein synthesis* (Herms & Mattson, 1992).

Very similarly to the carbon-nutrient balance hypothesis, the authors argue that *conditions favourable for growth are only a small subset of the conditions favourable for photosynthesis*. As an 'extension' of the carbon-nutrient hypothesis, the growth-differentiation hypothesis argues that *any factor that limits growth more than photosynthesis, such as moderate drought, moderate nutrient limitation or low temperature will increase the carbon pool available for allocation to secondary metabolism with little or no trade-off with growth. At moderate to high resource levels, however, the physiological trade-off becomes evident as a negative correlation between growth and secondary metabolism. In the cases of source limitation imposted by shade and sink limitation imposed by nutrient deficiency, the predictions of the growth-differentiation balance hypothesis are the same* (Herms & Mattson, 1992).

1.5.2 Status and critique of plant defence theory

All of the mentioned hypotheses have been experimentally tested many times; that is to say, all have been cited as the theoretical basis of many published articles (Stamp, 2003). Reviews of the accumulated published work show that evidence for and against the various theories is usually equivocal (e.g. Stamp, 2003; Koricheva, 2002b; Koricheva et al., 1998; Peñuelas & Estiarte, 1998; Herms & Mattson, 1992). For example, Koricheva et al. (1998) reviewed 147 experiments concerning the carbon-nutrient bal-

ance hypothesis conducted in the period from 1975 – 1997, leading to the result that overall, pooled carbon-based secondary metabolites, carbohydrates and phenylpropanoid derived compounds reacted as predicted, whereas other substances like terpenoids did not. Based on the many experiments and substances for which the outcome was not in line with the carbon nutrient balance hypothesis combined with some theoretical critique (e.g. Berenbaum, 1995; Edwards, 1992; Gottlieb, 1990), Hamilton et al. (2001) proposed to reject the carbon nutrient balance hypothesis. This suggestion led to an interesting but still rather inconclusive debate (Stamp, 2003; Nitao et al., 2002; Koricheva, 2002a; Lerdau & Coley, 2002): While some authors question the physiological and evolutionary assumptions of the theories (e.g. Koricheva, 2002a; Hamilton et al., 2001; Berenbaum, 1995; Edwards, 1992), others claim that many inconsistencies of experiments with theory are due to experimental shortcomings and that the theories for themselves are still useful (e.g. Stamp, 2003; Lerdau & Coley, 2002).

1.5.3 Some more fundamental considerations concerning changes in tannin concentrations

Despite an improved understanding of the biosynthetic pathway of condensed tannins and vigorous testing of the various plant defence hypotheses in recent years, a reliable prediction of changes in condensed tannin concentrations in response to plant development or an experimental treatment remains difficult. Reports of seasonal or developmental fluctuations of tannin concentrations in field grown *Lotus corniculatus*, for example, are contradictory between different studies and the responsible mechanisms are not well understood (Wen et al., 2003; Gebrehiwot et al., 2002; Roberts et al., 1993). The following section explores changes in tannin concentrations from a purely mathematical point of view and are bare of any biochemical or biological assumptions:

The tannin concentration $[CT]$ at the moment t is defined as the fraction between the absolute amount of tannin (T) found in a particular tissue and its corresponding biomass (B):

$$[CT] = \frac{T}{B} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \quad (1.2)$$

The tannin concentrations of a tissue of interest at any two points in time $t1$ and $t2$ ($t1 \neq t2$) can be written as:

$$[CT]_{t1} = \frac{T}{B} \qquad [CT]_{t2} = \frac{T + \Delta T}{B + \Delta B} \quad \ldots \ldots \ldots \ldots \ldots \ldots \quad (1.3)$$

Any change in tannin concentration between $t1$ and $t2$ is then defined as

$$\Delta\,[CT]_{t2-t1} = \frac{T+\Delta T}{B+\Delta B} - \frac{T}{B} = \frac{B\Delta T - T\Delta B}{B(B+\Delta B)} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1.4)$$

Because the biomass, B, is the sum of the dry weight of tannin, T, and of the dry weight of everything that is *not* tannin ($\bar{T}$), the biomass B can be expressed as

$$B = T + \bar{T} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1.5)$$

Thus, any change in the tannin concentration of the tissue of interest between $t1$ and $t2$ is given by

$$\Delta\,[CT]_{t2-t1} = \frac{\bar{T}\boldsymbol{\Delta}\mathbf{T} - T\boldsymbol{\Delta}\bar{\mathbf{T}}}{(T+\bar{T})(T+\bar{T}+\boldsymbol{\Delta}\mathbf{T}+\boldsymbol{\Delta}\bar{\mathbf{T}})} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1.6)$$

It can be seen that equation 1.6 is symmetric with regard to T and $\bar{T}$. This means that for the prediction of a change of the tannin concentration in a particular plant tissue, it is equally important to know the change in the amount of tannin (T) as it is to know the change in the amount of everything that is not tannin ($\bar{T}$). This observation is at odds with the common perception that changes of the tannin concentration of a given plant tissue are simply a function of tannin-synthesis.

Biologically, a change in T can result either from tannin synthesis or from tannin degradation. A change in $\bar{T}$ is more difficult to attribute to a certain chemical compound or group of compounds because it represents virtually *everything that is not tannin*; but in essence, $\bar{T}$ represents storage and growth processes. For example, the tannin concentration of a leaf can decrease or increase, respectively, due to either accumulation or export of non-structural carbohydrates (sugars and starch). Over longer periods of time, an enforcement of cell walls may increase $\Delta\bar{T}$ and decrease the tannin concentration in the tissue under investigation.

Equation 1.6 reveals that any change of the concentration of condensed tannins (or, of course, any other substance of interest) is completely predictable from the net rate of its biosynthesis provided that growth and storage processes are of no relevance. In the other extreme, it can be seen that changes of the tannin concentration can depend virtually on *everything but tannin-synthesis*, namely when growth and storage play important roles and the net synthesis rate of tannins is negligible. The failure to acknowledge

the importance of dilution processes – not just as an nuisance parameter but as active driver of tannin concentration dynamics – may be one of the major causes of the continued difficulty to predict fluctuations of tannin concentrations reliably (e.g. compare Wen et al., 2003; Gebrehiwot et al., 2002; Roberts et al., 1993). An assessment of the practical importance of tannin synthesis and dilution processes for the prediction of the tannin concentration in harvestable biomass shall be one of the main objectives of this thesis.

1.6 Aims and organization of this thesis

The research in this thesis is intended to provide essential plant scientific background knowledge for the application of tanniferous forage plants in agronomy for the control of gastrointestinal nematodes in ruminants and has two main goals:

1. It shall lead to an improved understanding of (fluctuations of) tannin concentrations of plant material – in particular of the harvestable aboveground biomass – in relation to the presence of plant 'enemies', the nutrient status and the developmental stage of the plant.

2. It shall explore the suitability of a variety of commercially available, tanniferous herbaceous species and cultivars for their use as antiparasitic agent in agronomy: candidate plant species[6] are *Onobrychis viciifolia* (sainfoin, dt. Esparsette), *Lotus corniculatus* (birdsfoot trefoil, dt. Hornklee), *Lotus pedunculatus* (big trefoil, dt. Sumpf-Schotenklee) and *Cichorium intybus* (chicory, dt. Chirorée).

The thesis is organized as follows: It begins with an exploration of the constitutive and elicitor-inducible tannin concentrations in leaflets of *Onobrychis viciifolia* in relation to the nutrient status and growth rate of the plant in Chapter 2. It then investigates the distribution of condensed tannins between plant organs in a total of six cultivars of three potentially suitable forage plant species in Chapter 3. This chapter also explores the tannin concentrations of harvestable aboveground biomass as a function of the developmental stage of the plants. Both Chapter 2 and Chapter 3 address tannin concentrations of tanniferous forage plants under exclusion of competition by other plant species. Chapter 4 describes a field experiment in which the agronomic suitability of tanniferous forages is evaluated and the seasonal development of tannin concentrations of harvestable aboveground biomass studied under field conditions including

[6] For pictures of the candidate plant species turn to page 183 (Appendix B).

competition with other (sown and unsown) plant species. Chapter 4 also links up with the module *Animal Nutrition* and the module *Parasitology* of the *Tannin-Project*; it includes an assessment of the palatability of tanniferous forage plants in comparison to a grass / legume mixture and an investigation of the antiparasitic efficacy of conserved *Onobrychis*. In Chapter 5, fresh tanniferous forages of known tannin concentrations were fed to sheep artificially infected with two important sheep parasites. The chapter aims at a first in-vivo dose-response curve of condensed tannins against gastrointestinal parasites. Chapter 6 deals with the administration of conserved tanniferous forage to sheep artificially infected with gastrointestinal nematodes. It explores whether the antiparasitic effect of tanniferous forage plants is still preserved in hay and silage. Finally, Chapter 7 contains a general discussion and aims at a synthesis of the entire thesis. It should be noted that Chapters 1, 2, 3 and 7 are the original work of the author of this thesis. Chapter 4, 5 and 6, however, are the result of cooperations between the different modules of the *Tannin-Project* – on the first page of each chapter, the contributions of the author of this thesis are specified. Also on the first page of each chapter it is specified whether the chapter is in preparation for publication, in press or has already been published.

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Chapter 2

Elicitor enhanced tannin concentrations at four levels of nutrient availability in *Onobrychis viciifolia*

HÄRING D.A.[12], HUBER M.J.[3],SUTER D.[1], EDWARDS P.J.[3], LÜSCHER A.[1]

Submitted to *Oecologia*

The current chapter investigates whether or not the tannin concentration of *Onobrychis viciifolia* can be stimulated by the simulated presence of plant enemies such as herbivorous insects or (pathogenic) bacteria and fungi. Furthermore, it was tested whether or not the constitutive and induced tannin concentration of *Onobrychis viciifolia* depends on the nutrient status and growth rate of the plant. The chapter is the original work of the author of this thesis.

[1]Agroscope Reckenholz-Tänikon Research Station ART, Zurich
[2]Institute of Plant Sciences, ETH Zurich
[3]Institute of Integrative Biology, ETH Zurich

2.1 Abstract

Condensed tannins (CT) play a key role in chemical plant defense against pathogens and herbivores. In a greenhouse experiment, we tested whether the tannin concentration of leaves of *Onobrychis viciifolia* increases in response to wounding, either alone or with the presence of a fungal, bacterial or insectan elicitor. The experiment was conducted with plants grown at four levels of P-availability (i.e. 0.0027, 0.075, 0.67, 2 mM P in the nutrient solution). There were three main hypotheses: (1) leaves respond to a sterile wounding treatment by increasing the production of condensed tannins, (2) this response is stronger when elicitors are applied to the wound, and (3) concentrations of condensed tannins, both constitutive and induced, are higher in plants grown at low as compared to high nutrient availability.

P-availability had a large effect on both mean relative growth rate and final biomass ($P < 0.001$), with the highest values of both being at 0.67 mM P. Independent of the wounding treatment, tannin concentrations decreased with increasing P-availability, from 94.9 to 69.0 mg g^{-1} leaf dry weight ($P < 0.001$). Contrary to expectation, sterile wounding reduced the tannin concentration, from 83.8 mg g^{-1} leaf dry weight in unscathed plants to 69.3 mg g^{-1} ($P < 0.01$); this was partly a dilution effect due to the presence of more non-structural carbohydrates in wounded leaves (160.8 v. 112.1 mg g^{-1}, $P < 0.05$). Local CT concentrations were higher when wounded leaves were treated with fungal, bacterial or insect elicitors (all elicitors; $P < 0.05$); however, only the insect elicitor (saliva of the lepidopteran *Spodoptera littoralis*) induced CT concentrations that were higher than those of unscathed leaves. Contrary to central predictions of some plant defense hypotheses, there was no evidence for stronger induction of CT's under low nutrient conditions, nor for a tradeoff between growth and defense.

Keywords:

proanthocyanidins, herbivores, pathogens, growth-defense tradeoff, *flg22*, *elf18*, *pen*, chitin, *Micrococcus lysodeikticus*, *Spodoptera littoralis*

2.2 Introduction

Condensed tannins are polyphenolic compounds with the ability to complex with metal ions and with macromolecules such as proteins and polysaccharides. They have antioxidative and radical scavenging properties (Haslam, 1996). Although their primary function in terms of evolutionary processes is disputable (Edwards, 1992), condensed tannins are certainly an important component of chemical plant defense: they have antibiotic activity against pathogens (Brownlee et al., 1990; Heil et al., 2002) and can deter herbivores (Bernays, 1981; Coley, 1986; Białczyk et al., 1999; Titus et al., 2000). Various studies have shown that the abundance and species richness of leaf-eating insects tends to be negatively correlated with foliar condensed tannin concentrations (Feeny, 1976; Coley, 1986; Białczyk et al., 1999; Forkner et al., 2004), and also that the central enzymes of their metabolic pathway can be induced by the real or simulated presence of pathogens or herbivores (Groten & Barz, 2000; Richard et al., 2000; Peters & Constabel, 2002; Rossi et al., 2004). Because of disciplinary divisions, there have been few comparative experiments using both pathogens and herbivores (Waterman & Mole, 1994). In this study, we investigated how the simulated presence of fungi, bacteria or herbivorous insects affects condensed tannin concentrations in the leaflets of *Onobrychis viciifolia*, using plants grown at four levels of phosphorus availability. We made use of recent discoveries of molecular structures by which plants recognize their opponents and reassessed central predictions of the controversially discussed 'plant defense theory' (Hamilton et al., 2001; Koricheva, 2002a,b; Lerdau & Coley, 2002; Stamp, 2003).

An important prerequisite of an effective plant defense is to discriminate between 'self' and potentially harmful 'non-self' (Medzhitov & Janeway, 2002). Plants may sense the presence of herbivores and pathogens by recognising distinctive molecular structures released by these organisms. Within minutes or hours after binding to specific receptors, such molecular structures (i.e. elicitors) can trigger early defensive reactions (Felix et al., 1999; Zipfel et al., 2006). Typically, these include an oxidative burst, extracellular alkalinisation, the production of the wound hormone ethylene, cell wall reinforcement and induction of various defense related genes (Groten & Barz, 2000). Later responses may be more specific, and involve the biosynthesis of allelochemicals (Cornelissen & Fernandes, 2001), protective enzymes (Salzer et al., 2000) and even the development of resistance against certain pathogens (Thuerig et al., 2006). The surveillance systems of plants are extremely sensitive and allow the recognition of elicitors even in concentrations as low as 10^{-12} – 10^{-9} M (Felix et al., 1999). They include chemoreceptors for elicitors that are characteristic for entire groups or classes of organisms, and

also receptors and mechanisms for recognising particular strains within these groups (Salzer et al., 2000). In this experiment, we used elicitors that are produced by many plant antagonists and that have been shown to activate plant defense responses in at least one other plant model system. The use of elicitors allowed us to simulate attack by a natural enemy, while making only small, standardized wounds that scarcely interfered with carbon assimilation.

To simulate fungal attack, we used a mixture of chitin and '*Pen*'. The polysaccharide chitin is an important component of the cell walls of all true fungi, and triggers defense reactions in species as unrelated as *Lycopersicon esculentum* (Fam. Solanaceae; Felix et al., 1999), *Arabidopsis thaliana* (Fam. Brassicaceae; Felix et al., 1999) and *Picea abies* (Fam. Pinaceae; Salzer et al., 1997). *Pen*, the second compound of our fungal elicitor mixture, was discovered recently in an aqueous extract of *Penicillium chrysogenum*, and has not yet been chemically identified (Thuerig et al., 2006). It is known to be recognized by tomato, tobacco and rice. In *Arabidopsis*, *Pen* was shown to provide resistance against several pathogens without having a direct antimicrobial effect. To simulate bacterial attack, we used a mixture of highly conserved bacterial peptides (i.e. *flg22* and *elf18*) and freeze-dried *Micrococcus lysodeikticus*; the most conserved domain of the bacterial flagellum protein 'flagellin', a peptide of 22 amino acids (i.e. *flg22*), is a strong elicitor in many Solanaceae as well as in *Arabidopsis* and rice (Felix et al., 1999). Additionally, we used the highly conserved domain of a procaryotic elongation factor (i.e. EF-Tu), a peptide of 18 amino acids (*elf18*; Kunze et al., 2004; Zipfel et al., 2006) and lyophilised *Micrococcus lysodeikticus*; the latter is thought to have elicitor activity due to the presence of cold-shock proteins (Felix & Boller, 2003). Finally, for the simulation of an insect attack, we used a well studied and powerful elicitor, 'volicitin' [N-(17-hydroxylinolenoyl)-L-glutamine], that is present in the oral secretion of *Spodoptera littoralis* larvae (Hoballah et al., 2002). As shown for *Zea mays* (Fam. Poaceae) and *Vigna unguiculata* (Fam. Fabaceae), volicitin is recognised by the plant and triggers the release of a blend of small, volatile terpenes that attract parasitoid wasps.

This study had three main objectives. The first was to test whether the simulated presence of pathogenic fungi, bacteria or herbivorous insects increases concentrations of condensed tannins, both locally and systemically. In this objective, we were interested in determining how *Onobrychis* responds to a broad range of biotic attacks, rather than understanding the response to particular elicitors in detail. The second was to test the hypothesis that tissues are defended in direct proportion to their 'value' (McKey, 1974). This hypothesis is based upon the idea that a given amount of tissue lost represents a

larger relative damage to a small, slow-growing plant than to a large, fast growing one (Coley et al., 1985). Thus, replacing tissues lost to herbivores and pathogens is likely to be more 'expensive' for a plant under low nutrient conditions, and we would expect such plants to produce higher concentrations of condensed tannins than those grown under high nutrient conditions. The third objective was to test a crucial assumption of several plant defense hypotheses, namely that carbon is 'cheap' at low but costly at high nutrient availability (Bryant et al., 1983; Coley et al., 1985; Herms & Mattson, 1992; Craine et al., 2003; Stamp, 2003). This assumption is based on the common finding that plants maintain photosynthetic activity under a wider range of environmental conditions than those under which growth is possible (Bryant et al., 1983; Herms & Mattson, 1992; Hoch et al., 2002); as a result, moderate sink limitation due to nutrient deficiency, drought or low temperatures can lead to the accumulation of carbohydrates and increased concentrations of secondary metabolites (Herms & Mattson, 1992). A rarely tested but central sub-hypothesis of this physiological model is that there is a tradeoff between growth and the production of secondary metabolites due to competition within the plant for common substrate and precursor molecules (e.g. phenylalanine). If such a tradeoff does indeed occur, it should be detectable as a negative correlation between growth and the concentration of secondary metabolites when plants are grown across a range of resource conditions (Herms & Mattson, 1992). Concerning the availability of nutrients, the prediction is that for a given level of nutrient availability, the correlation between growth (i.e. increase in biomass) and the concentrations of carbon based secondary metabolites such as condensed tannins should be more negative at moderate and high nutrient availability than at low nutrient availability. Many previous experiments addressing these issues have been criticized for containing only two levels of nutrient availability; such a design makes it difficult to determine the level of growth limitation and does not permit non-linear responses to be detected (Stamp, 2003). For these reasons, we chose to use four levels of nutrient availability (Herms & Mattson, 1992; Stamp, 2003). Because condensed tannins derive from the flavonoid pathway and make up a substantial fraction of leaf biomass (at least in the species we studied), they were particularly suitable for studying plastic responses of plant secondary metabolism to variations in resource availability (Reichardt et al., 1991; Herms & Mattson, 1992; Koricheva et al., 1998; Lerdau & Coley, 2002).

2.3 Material and Methods

2.3.1 Experimental design and plant material

In a greenhouse experiment, 120 plants of *Onobrychis viciifolia* (cv. Visnovsky, Fam. Fabaceae) were arranged in a split-plot design with 6 blocks (i.e. replicates) and 4 levels of phosphorus availability as the main-plot factor, and 5 elicitor treatments (including the two control treatments) as the sub-plot factor. Each main plot consisted of a container with 24 L volume and 920 cm^2 surface which was filled with quartz-sand (0.7 – 1.2 mm) and contained 5 experimental and 5 additional non-experimental plants. The experiment involved three phases: (i) an establishment period of two months when all plants were grown under the same conditions, (ii) a period of one month when plants were grown with one of four nutrient solutions differing in P-concentration, (iii) the treatment phase, in which plants were either left undamaged, or were wounded, or wounded and additionally treated with one of three possible elicitor-mixtures.

2.3.2 Phase 1: Establishment of experimental plants and nodulation

During the establishment period, plants were irrigated with 800 ml $container^{-1}$ day^{-1} of a full nutrient solution (Hammer et al., 1978), but with reduced concentrations of phosphorus (0.075 mM KH_2PO_4) and nitrogen (1.5 mM), as also done in Almeida et al. (1999). Between September and December, normal daylight was supplemented with artificial light from 6 am to 8 pm. The day / night temperatures were 22 / 15 °C and relative air humidity was 60 / 90 %.

To ensure that nodulation occurred in the artificial substrate, plants were inoculated with *Rhizobium sp.* derived from field grown plants of the same plant species and cultivar. For this purpose, clean root nodules were sterilized in isopropanol and then squeezed into a drop of water on yeast agar plates consisting of 1 g yeast, 10 g mannitol, 500 mg K_2HPO_4, 200 mg $MgSO_4$ $7{\cdot}H_2O$, 100 mg NaCl, 1 g $CaCO_3$ and 15 g agar per litre of distilled water (Vincent, 1970). Rhizobia cultures were left to grow in the dark at room temperature and plates were re-streaked when necessary. Rhizobia cultures were stored at 4 °C and plants were inoculated twice per week using suspensions of the cultivated Rhizobia.

By the end of the establishment period, the plants were 12.66 $\pm$ 0.40 cm tall, with an average biomass of 512 $\pm$ 31 mg dry weight and an average concentration of condensed tannin in their leaflets of 76.3 $\pm$ 3.2 mg CT g^{-1} dry weight.

2.3.3 Phase 2: Nutrient treatments

In phase 2, the uniform nutrient solution was replaced by four solutions with 0.0027, 0.075, 0.67 and 2 mM KH_2PO_4 (Almeida et al., 1999). The nitrogen concentration was maintained at 1.5 mM in all solutions, and all other nutrients were applied in the same concentrations as in the full nutrient solution (Hammer et al., 1978). Based on the results of Almeida et al. (1999) with *Trifolium repens*, the experimental P-concentrations were expected to impose a serious and a moderate limitation, respectively, on plant growth at the lowest two levels, the third level was expected to provide optimal conditions for growth, while the highest level was intended to provide an excess of phosphorus. Daily irrigation volumes and other growth conditions remained constant.

2.3.4 Phase 3: Wounding and elicitor treatments

Plants were wounded twice, nine and two days prior to harvest, and treated with an elicitor when appropriate. There were two control and three elicitor treatments. The control treatments included completely unscathed plants and plants that were wounded but not treated with an elicitor. The three elicitor treatments were intended to simulate attack by a broad range of either fungi, bacteria or leaf-eating insects. Therefore, we used elicitors and elicitor mixtures for which reactivity had been demonstrated in at least one other plant species. The 'fungal elicitor' was an aqueous solution containing 100 g ml^{-1} of hydrolized chitin (Salzer et al., 1997) and 100 g ml^{-1} *Pen* (Thuerig et al., 2006). The 'bacterial elicitor' contained 50 g ml^{-1} lyophilised *Micrococcus lysodeikticus* (Sigma; Felix & Boller, 2003) suspended in an aqueous solution of 1 M *flg22* (Felix et al., 1999) and 1 M *elf18* (Kunze et al., 2004; Zipfel et al., 2006). The 'insectan elicitor' consisted of freshly collected, undissolved regurgitant of *Spodoptera littoralis* caterpillars (Hoballah et al., 2002) that had been previously raised on *Onobrychis viciifolia* plants. Wounding was achieved by squeezing leaflets with sterile, grooved tweezers that left perforated marks of approximately 3.5 mm^2 $leaflet^{-1}$. On each of the plants to be wounded, three leaves were marked with a ribbon, and 15 leaflets from these leaves were damaged on two occasions (Fig. 2.1 on the following page). For plants assigned to an elicitor treatment, 3 L of the appropriate elicitor was applied to each wounded leaflet.

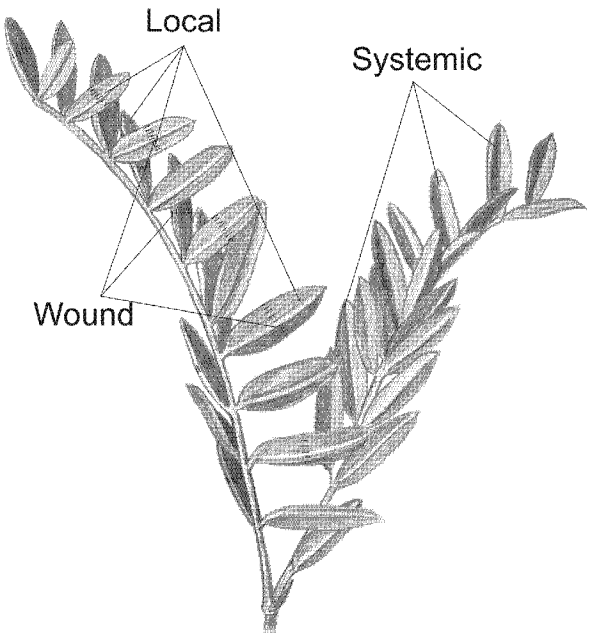

Figure 2.1: *Leaves of* Onobrychis viciifolia*. Local induction was measured in wounded leaflets. Systemic induction was measured in unwounded leaflets of an unwounded leaf on a wounded plant.*

2.3.5 Harvest and chemical analysis

To determine treatment effects on tannin concentrations, wounded leaflets (local) and comparable leaflets on unwounded leaves of the same plant (systemic) were collected and frozen (Fig. 2.1). This material was then lyophilized and ground to a fine powder using a ball mill. Roots were washed and dried with the other remaining tissues at 80 °C to determine the total biomass of each plant.

Condensed tannins were quantified photometrically in a butanol-hydrochloric-acid assay adapted from Terrill et al. (1992). Approximately 50 mg plant powder was three times extracted in teflon tubes using 5 mL of 7:3 (v / v) acetone / water with 1 g L^{-1} ascorbic acid and 4 mL diethyl ether. After each extraction, the tubes were centrifuged and the supernatants combined. The upper phase containing lipids and other non-polar molecules was discarded and the lower aqueous phase containing tannins was concentrated by rotary evaporation at 40 °C and 400 mbar. The resulting aqueous solution was made up to 20 mL with distilled water and the solid residue was stored at 4 °C for later use. A 1 mL aliquot of the aqueous solution was added to 6 mL of a freshly prepared butanol-hydrochloric-acid solution (950 mL BuOH and 50 ml HCL, 37 %) and heated

under reflux at 95°C for 75 min. The absorption of the so-called 'soluble tannins' was then measured at 550 nm. As shown in Terrill et al. (1992), a relatively large fraction of tannins is bound to proteins. This fraction was determined by a further extraction in which the solid residue was heated for 45 min at 95°C with 6 mL of a sodium-dodecyl-sulphate solution (10 mg of SDS, 50 mL mercaptoethanol, made up with distilled water to 1 L) and then centrifuged. This procedure was repeated and the combined supernatants were brought to a volume of 20 mL using the SDS solution described above. A 1 mL aliquot of the resulting solution was heated together with 6 mL of freshly prepared BuOH-HCL solution at 95°C for 75 min. The absorption was measured at 550 nm (protein-bound tannins). In order to relate optical densities to tannin concentrations, reference curves of extracted and purified tannins (Terrill et al., 1992) dissolved in either water (for the soluble tannins) or in SDS-solution (for the protein-bound tannins) were used. All concentrations of condensed tannins reported in this article refer to the sum of soluble and protein bound condensed tannins.

Non-structural carbohydrates (NSC), defined here as the sum of glucose, fructose, sucrose and starch, were analyzed in unscathed and in wounded leaflets as described in Wong (1990) and Körner et al. (1995). To do this, 10 mg plant powder were extracted in boiling water and centrifuged. The aqueous plant extract was then treated with isomerase and invertase, to convert fructose and sucrose into glucose which, after enzymatic conversion to gluconate-6-phospate (hexokinase reaction, hexokinase from Sigma Diagnostics, St. Louis, MO, USA), was photometrically quantified. The remainder of the water extract (including sugar and starch) was incubated with a dialysed crude fungal amylase ('Clarase' from *Aspergillus oryzae*, Enzyme Solutions Pty Ltd., Croydon South, VIC, AUS) for 15 h at 40°C to break down starch to glucose. Thereafter total glucose was determined as described above.

Phosphorus and nitrogen concentrations in the leaflets were determined using the pooled material for each block and level of P-availability. Phosphorus was analyzed after wet digestion of the plant powder with concentrated H_2SO_4 and H_2O_2 (Jones & Case, 1990) by colorimetric quantification of the blue molybdophosphate complex (Murphy & Riley, 1962). Nitrogen and carbon were quantified using a CHN-Analyzer (Euro EA 3000, HEKAtech GmbH, Weinberg, Germany).

2.3.6 Statistical analysis

Plant height, plant biomass, NSC data, and local and systemic CT concentrations were analyzed in split plot models (Gomez & Gomez, 1984) and the results summarized in ANOVA tables. Each model included coefficients for the blocks, for P-availability (main plot factor) and for the wounding and elicitor treatments (sub-plot factor). Additionally, the model contained coefficients for the interaction between the wounding and elicitor treatments and P-availability (interaction: P $\times$ T). To avoid a violation of the assumption of normally distributed residuals, it was necessary to *log*-transform the plant biomass data prior to their analysis, all other data were used untransformed.

In addition to the global F-tests summarized in ANOVA tables, more specific hypotheses were addressed by formulating linear contrasts between marginal means (i.e. between certain levels of P-availability or between certain wounding and elicitor treatments). Specific comparisons with regard to P-availability included the following: (1) the contrast between the response at the lowest and the highest level of P-availability, (2) the contrast between the response at the lowest and the third (and for growth optimal) level of P-availability, and (3) the contrast between the response at the third and the fourth level of P-availability. Specific comparisons with regard to the wounding and elicitor treatments included: (1) the contrast between unscathed and wounded plants (effect of the wound alone), (2-4) contrasts between the wounded plants and each elicitor treatment (test of the efficacy of the fungal, bacterial and insectan elicitor, respectively). Subsequently, each contrast was tested (t-test) using the estimates of the variances of the residuals suggested by the ANOVA models. The family-wise error rate (α = 0.05) was controlled for the tests concerning P-availability and the tests concerning the effect of the wound or the elicitors, using the p-value corrections suggested by Holm (1979).

For the elemental analyses, plant samples were pooled within each block and level of P-availability and data were analyzed in a two way ANOVA with the factors block and P-availability. Pairwise comparisons between the different levels of P-availability were subsequently made using Tukey HSD. All data analyses were performed in R (Version 2.4.0; www.r-project.org).

2.4 Results

2.4.1 Growth responses

The average total biomass of individual plants immediately before they were treated with one of four nutrient solutions was 512 $\pm$ 31 g dry weight. Thereafter, mean relative growth rates (ranked in order of increasing P-availability) were 8.0, 9.7, 28.0 and 21.1 % day^{-1}. By the end of the experiment, the plants in the four solutions differed considerably in size (Fig. 2.2), with an average height of 23.0, 25.6, 38.3 and 34.7 cm, respectively (ANOVA: $P < 0.001$), and an average dry weight of 2.15, 2.50, 6.24 and 5.05 g (ANOVA: $P < 0.001$). There were no significant differences in height or biomass between unscathed and wounded plants, and no differences among the various elicitor treatments (Tab. 2.1 on the next page). Thus, growth depended only on P-concentration in the nutrient solution, with the third highest level being the most favourable.

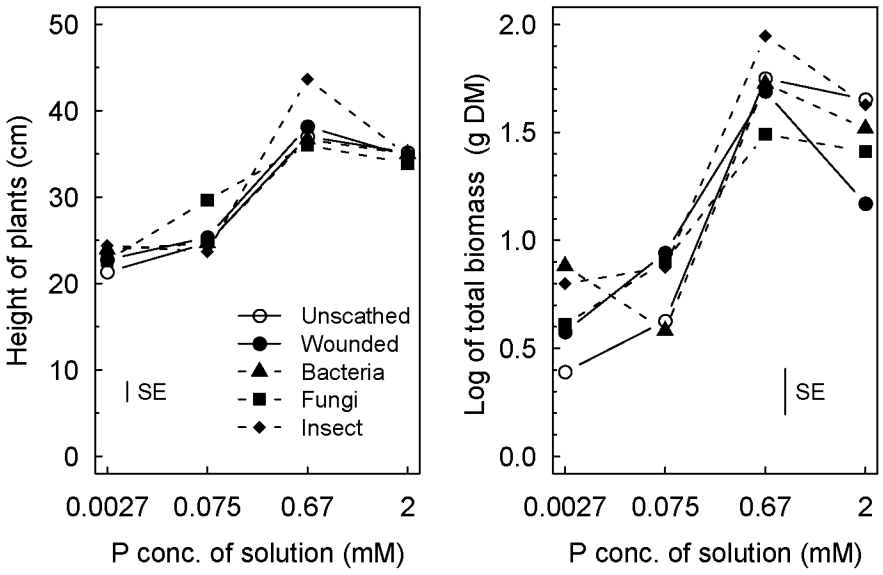

Figure 2.2: *Mean plant height (left) and total biomass (right;* log*-transformed) at the end of the experiment as a function of the phosphorus concentration in the nutrient solution and the applied wounding and elicitor treatment. Symbols and error-indicators refer to means and standard errors of the means (n = 6). For statistical details see Tab. 2.1 on the following page.*

Table 2.1: *Statistics of results: levels of significance for the global H_0-hypotheses (split-plot* ANOVA*s) and for the more specific linear contrasts including the relative change (Δ%).*

				Height		**Biomass**		**[CT]**$_{\text{Local}}$		**[CT]**$_{\text{Systemic}}$	
			Df	$P-value$		$P-value$		$P-value$		$P-value$	
ANOVA:											
Main plot:											
	Block		5	n.s.		n.s.		n.s.		n.s.	
	P		3	$***$		$***$		$*$		$*$	
	Residuals		15								
Sub-plot:											
	T†: Wounding & elicitors		4	n.s.		n.s.		$***$		n.s.	
	P $\times$ T		12	n.s.		n.s.		n.s.		n.s.	
	Residuals		80								
CONTRASTS:				P	Δ%	P	Δ%	P	Δ%	P	Δ%
	P1	→ P4	15	$***$	50.9	$***$	134.2	$*$	-17.3	$*$	-17.6
	P1	→ P3	15	$***$	66.4	$***$	189.6	n.s.	-11.4	n.s.	-11.2
	P3	→ P4	15	$*$	-9.3	$*$	-19.1	n.s.	-6.6	n.s.	4.0
	unscathed	→ wounded	80	n.s.	2.4	n.s.	-8.0	$*$	-17.3	n.s.	-7.3
	wounded	→ bacteria	80	n.s.	-0.6	n.s.	14.9	$*$	19.6	n.s.	-2.1
	wounded	→ fungi	80	n.s.	1.0	n.s.	3.5	$*$	15.9	n.s.	5.3
	wounded	→ insect	80	n.s.	4.7	n.s.	25.1	$***$	29.2	n.s.	0.6

†The test of T refers to the global F-test whether there are differences with regard of the target variable between any of the five wounding or elicitor treatments including the controls. Asterisks correspond to the p-values of the statistical tests: $***$ = P < 0.001, $**$ = P < 0.01, $*$ = P < 0.05, n.s. = not significant. For the linear contrasts, p-values were adjusted according to the method of Bonferroni as modified by Holm (1979), the first three and the last four contrasts share an $\alpha = 0.05$ each.

2.4.2 Phosphorus and nitrogen concentrations in leaflets

The phosphorus content of leaf tissue increased with increasing P concentration of the nutrient solution (ANOVA: $P < 0.001$; Fig. 2.3). Plants grown in the lowest P solution, and to a lesser extent those in the second lowest, showed typical symptoms of P-deficiency such as purple margins to leaflets and necrotic areas. Leaflet P concentration increased strongly and significantly from the second to the third level of P-availability, and also from the third to the highest level of P-availability. At 4.66 ± 0.30 mg P g^{-1} dry weight, concentrations at the highest level of P-availability were more than twice those at the lowest (2.19 ± 0.18 mg P g^{-1} dry weight). The nitrogen concentrations of leaflets increased slightly with increasing P-availability from 43.31 ± 1.05 mg P g^{-1} dry weight at the lowest level to 49.27 ± 2.61 mg P g^{-1} at the highest (Fig. 2.3; ANOVA $P < 0.05$). As a result, the N / P ratio of leaflets ranged considerably, from 19.8 at the lowest P-availability to 10.6 at the highest.

2.4.3 Non-structural carbohydrates (NSC)

Except for a small necrotic area around each wound, wounded leaflets remained green. To assess whether wounding had less visible effects, we measured the content of non-

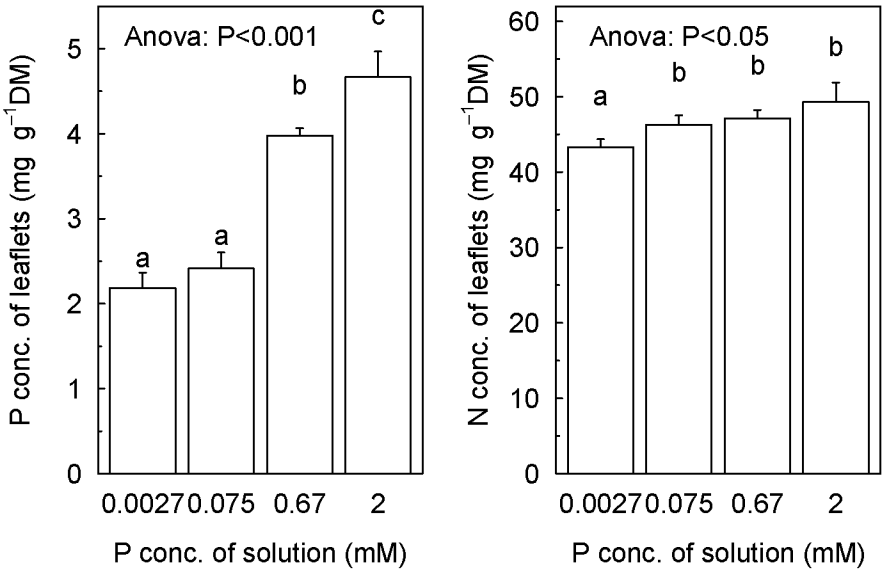

Figure 2.3: *Phosphorus and nitrogen concentration of the leaflets at the end of the experiment. Bars represent blockwise pooled samples (n = 6). Bars sharing a letter are not statistically different according to the Tuckey's honest significant difference (HSD).*

structural carbohydrates (i.e. the sum of glucose, fructose, sucrose and starch) in unscathed and in wounded leaflets (Fig. 2.4). Contrary to what we had expected, carbohydrate levels were higher in wounded leaflets than in unscathed ones ($P < 0.05$). Furthermore, the concentration of non-structural carbohydrates in unscathed leaves tended to decrease with increasing P-availability, although this result was not significant.

2.4.4 Concentrations of condensed tannins

The relationship between P-availability and CT concentrations in the absence of any wounding or elicitor treatment was assessed using the mean data for the unscathed control plants (in which no distinction can be made between local and systemic effects). The results show a strong and highly significant decline in tannin concentrations with increasing P-availability (94.9 ± 5.0, 84.9 ± 6.3, 75.5 ± 6.0 and 69.0 ± 3.6 mg g^{-1} dry weight, respectively; ANOVA: $P < 0.001$). The wounded plants also showed a significant decrease in tannin content with increasing P-availability, both in the damaged leaflets and in leaflets of undamaged neighbouring leaves ($P < 0.05$ in both ANOVAs; Tab. 2.1 on page 36; Fig. 2.5 on the next page).

Wounding and the application of elicitors affected tannin concentrations locally (i.e. in

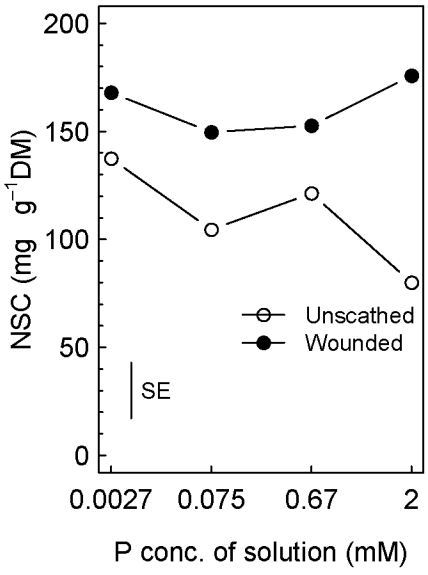

Figure 2.4: *Non-structural carbohydrates (NSC) in unscathed and wounded leaflets. Symbols and error-indicators refer to means and standard errors of the means (n = 6).*

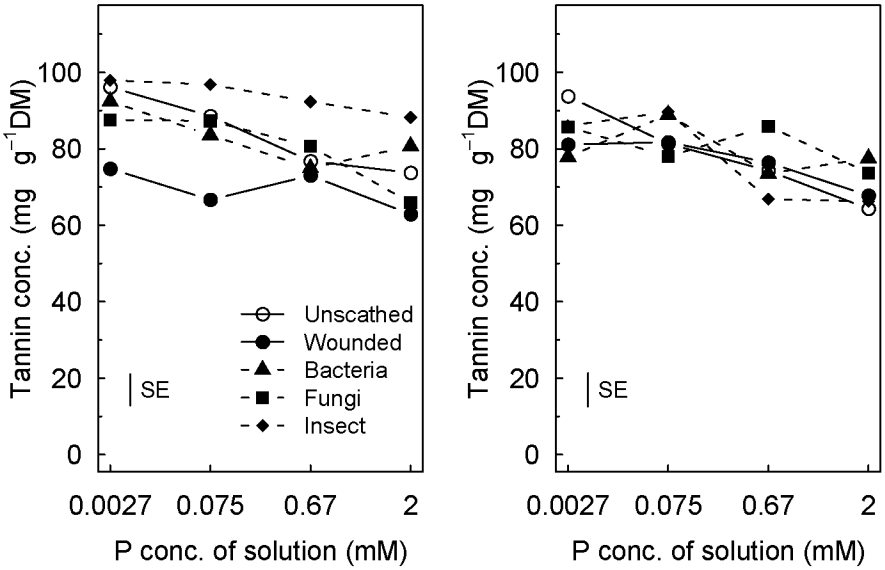

Figure 2.5: *Mean local tannin concentrations (left) and mean systemic tannin concentrations (right) as a function of the phosphorus concentrations in the nutrient solution and the applied wounding and elicitor treatments. Symbols and error indicators refer to means and standard errors of the means (n = 6). For statistical details see Tab. 2.1 on page 36.*

damaged leaflets; ANOVA local: $P < 0.001$) but not systemically (i.e. leaflets of undamaged neighbouring leaves; ANOVA systemic: $P = 0.964$; Fig. 2.5). Tannin concentrations of leaflets that were wounded but not treated with an elicitor were lower than those in leaflets of unscathed plants ($P < 0.01$). However, tannin concentrations of leaflets that had also been treated with one of the elicitors were significantly higher than those of leaflets that had only been wounded (all elicitors: $P < 0.05$; Tab. 2.1 on page 36). For plants treated with either the bacterial or the fungal elicitor this difference was not large and the CT concentrations were similar to those of unscathed plants. The only elicitor that increased tannin concentrations above the level of unscathed leaves was saliva of *Spodoptera littoralis* larvae. There was no evidence for a stronger stimulation of tannin concentrations by the wounding or elicitor treatments at low as compared to high nutrient availability (no significant P $\times$ T interaction; Tab. 2.1 on page 36).

It is generally supposed that producing tannins at low nutrient availability is 'cheap' because there is a surplus of carbon, whereas at moderate to high levels of nutrients, tannin synthesis is costly because it diverts carbon resources from plant growth (growth-defense tradeoff). We therefore expected to find that as P-availability increased there would be an increasingly negative correlation between plant biomass and the tan-

nin concentrations of unscathed plants. However, Pearson regression coefficients for this relationship, ranked from low to high P-availability, were -0.39, 0.50, 0.32 and 0.49, respectively; they were in no case significant.

2.5 Discussion

Onobrychis viciifolia responded to the experimental range of phosphorus availabilities with large differences in relative growth rate and chemical composition. The increasing phosphorus concentration in the nutrient solution was reflected in the P-concentration of the plant and – due to its feedback effect on nitrogen fixation (Almeida et al., 1999) – also in the N concentration of the leaves. At the lowest level of P-availability, plants showed typical deficiency symptoms, and growth was reduced compared to that of plants at higher P concentrations. At the second, third and fourth levels, P-availability was sub-optimal, optimal and super-optimal for growth, respectively. The N / P ratio of plant tissue is usually a valuable indicator of nutrient limitation and saturation (Tessier & Raynal, 2003): an N / P ratio of 13 – 17 suggests a balanced supply of both N and P, while lower or higher values indicate P and N limitation, respectively. Leaves in the low P-treatment had an N / P ratio of 19.8, clearly indicating strong P limitation. However, the N / P ratio of 10.6 at the highest P-availability should not be interpreted as an indicator of N limiting conditions; given the vigour of growth together with N concentration of almost 50 mg N g^{-1} dry weight in leaves suggests that the plants had plenty of nitrogen (N_2-fixation) but indicates an excessive uptake of phosphorus under these unnaturally P-rich conditions (Almeida et al., 1999).

The lack of a difference in total biomass between unscathed and wounded plants at the end of the experiment, as well as the higher content of non-structural carbohydrate in wounded as compared to unscathed leaflets, suggest that the small and standardized wounds used in this experiment did not reduce carbon assimilation. The higher carbohydrate status of wounded leaflets may have partially resulted from an enhanced import of assimilates from source leaves, as demonstrated in a C^{13}-labelling experiment with wounded leaves of *Populus* (Arnold & Schultz, 2002). In fact, in *Populus* saplings it was possible to stimulate higher concentrations of condensed tannins in developing sink leaves but not in fully developed source leaves. The authors concluded that induction of tannin synthesis requires sink activity and the associated import of carbon from source leaves. Both the increased import of assimilates described by Arnold & Schultz (2002) and the higher carbohydrate status of wounded leaflets measured in our experi-

ment may represent an early condition of heightened alert; subsequently, the presence of an elicitor may enable the plant to recognise the antagonist, triggering conversion of these carbohydrates into the appropriate chemical defenses.

2.5.1 Induction of secondary metabolism

In our experiment, the application of the regurgitant of *Spodoptera littoralis* caterpillars stimulated tannin concentrations in wounded leaflets of *Onobrychis viciifolia* beyond the level reached by any of the other treatments. With respect to a potential herbivore, such a local increase in the concentration of condensed tannins may have a physiological effect after ingestion, but is more likely to cause the insect to move and feed elsewhere (Bernays, 1981; Edwards & Wratten, 1983). The induction of condensed tannins by *Spodoptera* saliva is consistent with several other studies; for example, Rossi et al. (2004) found that the protein binding capacity of leaves of *Quercus myrtifolia* that had been mined or chewed by insects was 21 to 25 % higher than that of undamaged leaves. It is also in line with the repeated finding in various plants of a stimulated expression of enzymes of the phenyl-propanoid and flavonoid pathways by the real or the simulated presence of herbivores or pathogens. Ralph et al. (2006) for example, detected up-regulation of chalcone synthase (CHS), phenylalanine-ammonia lyase (PAL) and dihydroflavonol reductase (DFR), all of which are key enzymes in the biosynthetic pathway of condensed tannins[4], in response to mechanical wounding, the presence of budworms (*Choristoneura occidentalis*) or weevils (*Pissodes strobi*) in *Picea sitchensis* by means of cDNA microarrays. Similar transcriptional and enzymatic up-regulations in response to the real or simulated presence of herbivorous insects have also been reported in *Picea glauca* (Richard et al., 2000) and *Populus tremuloides* (Peters & Constabel, 2002).

The presence of fungal or bacterial elicitors enhanced the tannin concentrations in the local surrounding of the wound compared to leaflets that were wounded but not treated with an elicitor. This finding is in accordance with a higher PAL activity and an increasing concentration of phenolic metabolites after the application of fungal or bacterial elicitors in the culture medium of different soybean cultivars (Groten & Barz, 2000). However, in our experiment, the microbial elicitors were clearly less effective than the caterpillar saliva and did not stimulate tannin concentrations beyond the level of unscathed leaflets.

[4] See Fig. 1.1 on page 3

2.5.2 No interaction between P-availability and induced defense and no evidence for a growth-defense tradeoff

We found no evidence for a relationship between the induction caused by any of the elicitors used in this study and the nutrient level to which the plants were exposed (Tab. 2.1 on page 36). The lack of a stronger induction at low as compared to high nutrient availability, despite an apparent recognition of at least the insectan elicitor, seems incompatible with the common assumption that at low nutrient availability 'cheap' carbon would feed into the production of carbon based secondary metabolites (Bryant et al., 1983; Coley et al., 1985; Herms & Mattson, 1992; Stamp, 2003). The fact that biomass production varied by a factor of nearly three according to P-availability demonstrates the impressively large potential for carbon acquisition in excess of growth demands under some conditions. However, it appears that the relative availability of growth limiting nutrients and carbon is not a good indicator for the degree of induction – perhaps because carbon is 'cheap' even at high nutrient availability (Craine et al., 2003), or perhaps because the accumulation of condensed tannins at low nutrient availability is somehow constrained.

Independent of the wounding or elicitor treatment, tannin concentrations decreased with increasing nutrient availability or growth rates and with decreasing C / N ratios, respectively. All of these observations appear to be compatible with the predictions of the carbon-nutrient balance or the growth differentiation hypotheses (Bryant et al., 1983; Herms & Mattson, 1992; Stamp, 2003), and in previous experiments such results have often been interpreted as evidence for these hypotheses. However, negative correlations across nutrient availabilities can result from passive dilution as well as from a tradeoff between growth and defense (Koricheva, 1999; Häring et al., 2007). Moreover, these hypotheses have been based, at least in part, on the observation that slow growing plants of nutrient poor sites often contain higher concentrations of tannins and other putatively defensive carbon based secondary metabolites than fast growers on fertile soils (Bryant et al., 1983; Coley et al., 1985; Herms & Mattson, 1992); this same observation should not also be used as evidence in support of the hypotheses.

In this study, correlations between growth and tannin concentrations at any one level of P-availability were always weak and never significant. With regard to their sign, the correlation coefficients were negative at the lowest level of P-availability but positive at moderate to high nutrient availability, just the opposite what the growth-differentiation balance hypothesis would predict. Thus, our results contradict the idea of an 'inevitable'

tradeoff between the production of tannins and growth (Herms & Mattson, 1992), but rather suggest that the plants growing most efficiently at a particular level of nutrient availability can also be those with the highest concentrations of tannins. Although a recent meta-analysis confirmed the general assumption of fitness costs of antiherbivore defenses, it was concluded that the experimental evidence is inconsistent with the notion that fitness costs are incurred through diversion of common limited resources (Koricheva, 2002b). As an alternative, it was suggested that fitness costs of defense arise through interactions between plants and their environments rather than through tradeoffs for resources. Although carbohydrates and other storage related compounds tend to accumulate when environmental conditions limit growth but not photosynthesis (Koricheva et al., 1998; Hoch et al., 2002; Hoch & Körner, 2003; Häring & Körner, 2004), this does not necessarily mean that the surplus carbon feeds into the pool of secondary metabolites (Koricheva et al., 1998; Häring & Körner, 2004).

2.5.3 Conclusions

Concentrations of condensed tannins in leaflets of *Onobrychis viciifolia* can be enhanced by the presence of molecular structures originating from potential enemies independent of the nutrient status of the plant. In this study, the saliva of the herbivorous insect *Spodoptera littoralis* stimulated local tannin concentrations more effectively than microbial elicitors. Overall, our results are compatible with the hypothesis that chemical defense should decrease when enemies are absent and increase when they are present (McKey, 1974; Stamp, 2003). They contradict, however, the common assumption that under low nutrient conditions surplus carbon feeds into the production of carbon based secondary metabolites, while at high nutrient availability there is a tradeoff between growth and defense. Our data suggest that tradeoffs for resources between growth and defense are at least less general than predicted by the growth-differentiation balance hypothesis.

2.6 Acknowledgements

We are very grateful to Th. Boller, G. Felix and P. Salzer from the University of Basel who generously provided us with elicitors and to T. Turlings from the University of Neuchâtel who provided the *Spodoptera* caterpillars. We thank Ch. Staehelin from the University of Geneva and U. Merz from ETH Zurich for their help with the cultivation of Rhizobia.

We thank G. Hoch from the University of Basel for the NSC-analysis and O. Hugueneni-Elie, J. Leifeld and H.R. Bosshard from our own laboratories for their support with the analytical procedures. The project was financed by the Swiss Federal Office for Agriculture.

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Annals of Botany **99**: 111–120, 2007
doi:10.1093/aob/mcl227, available online at www.aob.oxfordjournals.org

Biomass Allocation is an Important Determinant of the Tannin Concentration in Growing Plants

D. A. HÄRING[1,2], D. SUTER[1,*], N. AMRHEIN[2] and A. LÜSCHER[1]

[1]*Agroscope Reckenholz-Tanikon, Research Station ART, Reckenholzstrasse 191, 8046 Zurich, Switzerland and* [2]*Institute of Plant Sciences, ETH Zurich, 8092 Zurich, Switzerland*

Received: 24 July 2006 Returned for revision: 11 August 2006 Accepted: 1 September 2006

• *Background and aims* Condensed tannins (CTs) in the diet affect consumers in a concentration-dependent manner. Because of their importance in plant defence against herbivores and pathogens as well as their potential application against gastrointestinal parasites of ruminants in agronomy, an understanding of the seasonal dynamics of CT concentrations during plant growth is essential.
• *Methods* Over a vegetation period, CT concentrations in leaves, stems and roots and the biomass proportions between these organs were investigated in *Onobrychis viciifolia*, *Lotus corniculatus* and *Cichorium intybus*. Based

Chapter 3

Biomass allocation is an important determinant of the tannin concentration in growing plants

HÄRING D.A.[12], SUTER D.[1], AMRHEIN N.[2], LÜSCHER A.[1]

Published in *Annals of Botany* 99 (1): pp. 111-120, 2007.

The current chapter investigates the distribution of condensed tannins within three tanniferous (forage) plant species. It then emphasizes the importance of shifts in biomass allocation to plant organs with different tannin contents during plant development for the determination and prediction of the tannin concentration in harvestable aboveground biomass of tanniferous forage plants. The chapter is the original work of the author of this thesis, from the experimental planning to the writing of the article.

[1]Agroscope Reckenholz-Tänikon Research Station ART, Zurich
[2]Institute of Plant Sciences, ETH Zurich

3.1 Abstract

- Background and aims: Condensed tannins (CT) in the diet affect consumers in a concentration dependent manner. Because of their importance in plant defence against herbivores and pathogens as well as for their potential application against gastro-intestinal parasites of ruminants in agronomy, an understanding of seasonal dynamics of CT concentrations during plant growth is essential.

- Methods: Over a vegetation period, CT concentrations in leaves, stems and roots and the biomass proportions between these organs were investigated in *Onobrychis viciifolia*, *Lotus corniculatus* and *Cichorium intybus*. Based on the experimental data, a model has been suggested to predict CT concentrations in harvestable biomass of these species.

- Key Results: During the experiment, leaf mass fractions of plants decreased from 85, 64, 85 to 30, 18, 39 % DW in *Onobrychis*, *Lotus* and *Cichorium*, respectively, and proportions of stems and roots increased accordingly. While CT concentrations almost doubled in leaves in *Onobrychis* (52 to 86 mg g^{-1} DW, $p<0.001$) and *Lotus* (25 to 54 mg g^{-1} DW, $p<0.001$), they were stable at low levels in expanding leaves of *Cichorium* (5 mg g^{-1} DW) and in stems and roots of all investigated species. Due to an inverse effect of the increasing CT concentrations in leaves and simultaneous dilution from increasing proportions of 'CT-poor' stems, CT concentrations in harvestable biomass were stable over time in all investigated species: 62, 26, 5 mg g^{-1} DW for *Onobrychis*, *Lotus* and *Cichorium*, respectively.

- Conclusions: As a consequence of the unequal distribution of tannins in different plant parts and due to the changing biomass proportions between them, various herbivores (e.g. a leaf-eating insect and a grazing ruminant) may not only find different concentrations of CT in their diets but also different CT dynamics during the season. For the prediction of seasonal variations of CT concentrations, biomass allocation and accumulation of none-CT plant material are likely to be as important predictors as the knowledge of CT synthesis and its regulation.

Keywords

Onobrychis viciifolia, *Lotus corniculatus*, *Cichorium intybus*, condensed tannin, proanthocyanidins, seasonal dynamics, forage, plant defence, concentration, biomass allocation, secondary metabolism, model.

3.2 Introduction

Tannins are phenolic plant secondary metabolites and are involved in plant-pathogen (Brownlee et al., 1990; Edwards, 1992; Heil et al., 2002) and plant-herbivore interactions (Bernays, 1981; Min et al., 2003; Forkner et al., 2004). While different pathogens or herbivores show varying levels of tolerance towards tannins, there generally is a threshold for a given organism above which tanniferous diets are repellent or have detrimental effects on a plant's opponent (Bernays, 1981; Brownlee et al., 1990; Waterman & Mole, 1994; Heil et al., 2002). Thus, tannins rarely are acutely toxic, but act in a concentration-dependent manner. In animal husbandry, high dietary concentrations of tannins are known to reduce the digestibility of the fodder and to adversely affect the ruminant's health (Min et al., 2003). However, at moderate concentrations ($<$50 mg g^{-1} DW), condensed tannins can reduce the risk of bloat, increase the uptake of essential amino acids and proteins, enhance the production of milk and wool and be effective against gastro-intestinal parasites (Min et al., 1999; Barry & Mcnabb, 1999; Aerts et al., 1999; Athanasiadou et al., 2001; Niezen et al., 2002; Marley et al., 2003; Paolini et al., 2004; Hoste et al., 2006; Mueller-Harvey, 2006). Because of the importance of tannins in plant defence and for the health of ruminants, an understanding of the dynamics of CT concentrations in growing plants is essential.

For the last 30 years, it has been a major goal in ecology to find a uniform plant defence theory (Rhoades & Cates, 1976; Stamp, 2003). Until recently, the carbon-nutrient balance hypothesis (CNB; Coley et al., 1985; Bryant et al., 1983) and the growth-differentiation hypothesis (GDB; Herms & Mattson, 1992) were the most influential theories for the prediction of phenotypic variations in concentrations of secondary metabolites. The CNB predicts that the concentrations of carbon based secondary metabolites (secondary metabolites that consist only of C, O and H) will increase when the nutrient availability limits growth more than photosynthesis. The GDB assumes a trade-off between growth and the production of secondary metabolites, predicting that any environmental factor that slows growth more than photosynthesis will increase the resource pool available for allocation to secondary metabolites. Hence, the predictions of the CNB hypothesis are a subset of the predictions of the GDB hypothesis (Herms & Mattson, 1992). It has been derived from the GDB hypothesis that periods of strong growth should correlate to low allocation for defence (Lerdau et al., 1994).

Although appealing with regard to their simplicity and generality, today's experimental

evidence suggests that these hypotheses are not adequate to predict levels of individual secondary metabolites (see Koricheva et al., 1998; Hamilton et al., 2001; Nitao et al., 2002). It was concluded that the prediction of concentrations of secondary metabolites requires a shift to models with a mechanistic basis in physiology and biochemistry (Berenbaum, 1995; Hamilton et al., 2001; Koricheva, 2002). Especially in strongly expanding tissues - e.g. in leaflets of *Onobrychis viciifolia* from the earliest unfolded stages to maturation of the leaves (Joseph et al., 1998; Koupai-Abyazani et al., 1993), or in very heterogeneous plant material (e.g. in harvestable biomass of *Lotus corniculatus*: Roberts et al., 1993; Gebrehiwot et al., 2002; Wen et al., 2003) - previous studies on the dynamics of CT concentrations yielded controversial results. The mechanisms driving CT concentration dynamics are not well understood.

It is thought that a central aspect of the difficulty to predict changes of tannin concentrations in growing plants and to integrate biochemical knowledge into models is the fact that secondary metabolites rarely are uniformly distributed throughout the plant. They occur more concentrated in some organs, tissues, cells or ducts than in others. Therefore, changes in the biomass allocation to different parts of the plant can alter the overall secondary metabolite concentration of plants or of harvestable plant parts even though the concentrations within particular parts remain constant. Thus, we suggest modelling concentrations of secondary metabolites, in particular of constitutive compounds such as tannins, from terms modelling the physiological responses within relatively homogeneous compartments and from terms modelling changes in the biomass allocation between them.

The major aim of the experiment presented here was to create a framework that allows to model the seasonal dynamics of tannin concentrations in the harvestable biomass of herbaceous, tanniferous plant species with agronomical value. In an outdoor experiment, the seasonal and ontogenetic dynamics of biomass allocation to leaves, stems and roots, and of CT concentrations in these organs, were investigated in six cultivars of three tanniferous plant species during a vegetation period.

3.3 Material & Methods

This outdoor experiment was conducted in artificial microswards at Agroscope Reckenholz-Tanikon, Research Station ART, in Zurich, Switzerland (47° 25' N, 8° 31' E). Three

tanniferous plant species with two cultivars each - i.e. *Onobrychis viciifolia* (sainfoin; cv. Visnovsky and commercial seed), *Lotus corniculatus* (birdsfoot trefoil; cv. Oberhaunstädter and Lotar) and *Cichorium intybus* (chicory; cv. Puna and Lacerta) - were selected as experimental plants. These three plant species have been chosen because they are considered as potentially valuable forage plants in agronomy and are currently investigated for their beneficial effects on ruminants. All of them are also consumed by insects. The allocation of biomass and condensed tannins to leaves, stems and roots was studied seven times in intervals of two to three weeks during the course of the vegetation period in 2003 (from sowing at the end of May until leaf senescence in October).

3.3.1 Design and growth conditions

Each cultivar was grown in monoculture in square pots with a volume of 12 litres and a surface of 480 cm^2 at a density of five plants per pot. The soil was mixed from two parts of potting soil and one part of loamy field soil. A total of 126 experimental pots (3 species x 2 cultivars x 7 harvests x 3 replicates) were arranged in a split plot design with 'species' as the main plot factor and 'time of harvest' as the sub-plot factor. Within each block, pots of a given species were positioned randomly and in close proximity to each other to form artificial microswards, and then surrounded by additional non-experimental pots of the same species, used to prevent border effects.

During the experiment, plants were exposed to outdoor conditions i.e. mean daily temperatures increased from 15°C in May to 22°C in July and then decreased again to 6°C in October. Daylength increased from 15.5 h in May to 16 h in June and decreased to 11 h in October. No additional fertilizer was provided but when necessary, plants were watered early in the morning.

In seven successive, destructive harvests, one pot per block and cultivar was selected at random. After each harvest, pots within each artificial sward were moved together in order to ensure that neighbouring plants of the harvested pots did not experience competitive advantages.

3.3.2 Harvests

The heights of all plants in each pot selected for harvest were measured. Plants were then cut off at 5 cm above ground level. This 'harvestable biomass' was separated into leaves and stems and the total leaf area per pot was determined. Roots were washed free of soil. Leaves, stems and roots respectively were dried at 60°C for 48 h and then weighed. For the calculation of total biomass and leaf-, stem- and root mass fraction of the entire plants, stubbles (biomass 0 – 5 cm) were also separated into leaves and stems, dried and weighed as mentioned above. Mean specific leaf area (SLA) per pot was calculated as the total leaf area divided by the corresponding leaf dry weight. Leaves, stems and roots were ground to pass a 0.75 mm sieve and analysed separately for CT using a butanol-HCL assay (see below). Tannin concentrations in the harvestable biomass and on the level of the whole plant were calculated from tannin concentrations measured in leaves, stems and roots and from the relative contributions of these organs to the respective biomass.

3.3.3 Tannin analysis

We used a modified version of the butanol-HCL assay described in Terrill et al. (1992) which had been adapted from Porter et al. (1986). Dried and ground plant material (100 mg) was extracted three times with a mixture of 5 ml 7:3 (v/v) acetone/water with 1 g L^{-1} ascorbic acid and 4 ml of diethyl ether in teflon tubes. After each extraction, the tubes were centrifuged and the supernatants combined. The upper phase, containing pigments, lipids and other non-polar molecules was discarded and the lower phase containing tannins was concentrated by rotary evaporation at 40°C and 400 mbar. The resulting aqueous solution was filled up to 20 ml with distilled water and the solid residue was stored at 4°C for later use. One ml of the obtained aqueous solution was added to 6 ml of freshly prepared BuOH-HCL solution (950 ml BuOH, 50 ml HCL 37 %) and heated under reflux (95°C for 75 min). Finally, the absorption of the so-called soluble tannins was measured at 550 nm.

As shown in Terrill et al. (1992) a relatively large fraction of tannins is bound to proteins, which was analysed as follows: The solid residue from the extraction described above and 6 ml of a SDS-solution (10 mg sodium-dodecyl-sulfate, 50 ml of mercaptoethanol filled up with distilled water to a volume of 1 L) was heated at 95°C for 45 min and then centrifuged. This extraction was repeated once more and the combined supernatants were brought to a volume of 20 ml using the SDS-solution (see above). One ml of the

resulting solution was heated together with 6 ml of freshly prepared BuOH-HCL solution and the absorption measured at 550 nm (protein-bound tannins).

In order to relate the optical densities to tannin concentrations, reference curves were obtained using purified *Lotus pedunculatus* tannin as in Terrill et al. (1992). Pure BuOH-HCL solution (950 ml BuOH, 50 ml HCL 37 %) was used as blank; see discussion in Terrill et al. (1992). All results are reported as the sums of the soluble and the protein-bound fractions.

3.3.4 Statistical analysis and model interpretation

Data were analysed separately for each species using multiple linear regression models. We put the emphasis on easy model interpretation and model consistency across species rather than on the parametric simplicity of the individual models. Therefore, we used untransformed response variables and centred explanatory variables. Since the explanatory variable 'time' (t) was centred on its mean, the letter t in the equations refers to the number of weeks after sowing minus 11.29. Thus, the intercepts of the linear regression models can be interpreted as the estimated mean response in mid-season, at the maturity of the plants.

To model the growth dynamics of the plants (biomass: B; Eqn. 3.1), typical sigmoidal curvatures were assumed for all cultivars. Hence, we allowed the linear models to adapt a polynomial fit of 3rd degree for the temporal structure rather than simple lines for any of the three species. Furthermore, we assumed the growth dynamics to be characteristic for a certain species and allowed the curves of two cultivars of the same species to differ in the intercept only (no interaction terms).

$$B(c,t) = \beta_0 + \beta_1 \cdot c + \beta_2 \cdot t + \beta_3 \cdot t^2 + \beta_4 \cdot t^3 + \varepsilon \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3.1)$$

where

- $\varepsilon \sim \mathcal{N}(0, \sigma_\varepsilon^2)$
- $c = 0$, for cultivars Visnovsky, Lotar and Lacerta and
  $c = 1$, for cultivars commercial seed, Oberhaunstädter, Puna
- t represents the time in weeks (centered on its mean)

- $\beta_0, \beta_1, \beta_2, \beta_3$ and β_4 are the coefficients of the regression model.

The leaf mass fraction of the harvestable biomass (LMFH: L; Eqn. 3.2) was modelled analogously to the model for biomass:

$$L(c,t) = \lambda_0 + \lambda_1 \cdot c + \lambda_2 \cdot t + \lambda_3 \cdot t^2 + \lambda_4 \cdot t^3 + \varepsilon' \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3.2)$$

where

- $\varepsilon' \sim \mathcal{N}(0, \sigma^2_{\varepsilon'})$
- $c \in \{0, 1\}$ (see Eqn. 3.1 on the previous page)
- t represents the time in weeks (centered on its mean).
- $\lambda_0, \lambda_1, \lambda_2, \lambda_3$ and λ_4 are the coefficients of the regression model.

In the models for biomass and LMFH, the dependences of the response variables from time (i.e. $\beta_2, \beta_3, \beta_4$ or $\lambda_2, \lambda_3, \lambda_4$ combined) were tested using partial F-tests between the full and the reduced models.

Tannin concentrations (T; Eqn. 3.3) of leaves, stems, roots, harvestable biomass and of entire plants were modelled as linear functions of time (there was no evidence for temporal dynamics of higher order) including an interaction term for the slope of the two cultivars per species.

$$T(c,t) = \tau_0 + \tau_1 \cdot c + \tau_2 \cdot t + \tau_3 \cdot c \cdot t + \varepsilon'' \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3.3)$$

where

- $\varepsilon'' \sim \mathcal{N}(0, \sigma^2_{\varepsilon''})$
- $c \in \{0, 1\}$ (see Eqn. 3.1 on the previous page)
- t represents the time in weeks (centered on its mean).
- τ_0, τ_1, τ_2 and τ_3 are the coefficients of the regression model.

For the two cultivars of each species, the term $(\tau_2 + \tau_3 \cdot c)$ can be interpreted as the estimated weekly change of tannin concentration (mg CT g^{-1} DW $week^{-1}$) during the

experiment.

Some models, especially the models for biomass, indicate some heterogeneity of the variance of the residuals; however, these deviations from the assumption of homogeneous variances seemed acceptable compared to the otherwise more difficult interpretation of models involving variance-stabilising transformations. Dots in diagrams refer to values per pot and allow to appreciate the fit of the models which are displayed as lines. For a better visualisation in diagrams, dots were slightly jittered along the x-axis. The estimated coefficients of the models in equations one to four and the corresponding tests of significance are summarized in tables. All statistical analyses have been conducted using R (Version 2.1.0; www.r-project.org).

3.4 Results

3.4.1 Growth and ontogenesis

All six cultivars were observed from an early developmental stage (4 weeks after sowing) to the onset of senescence in autumn, 20 weeks after sowing. At the first harvest, average plant heights of all cultivars measured between 8 and 12 cm and average biomasses were below 10 g DW per pot. At the end of the experiment, plants had reached average heights between 40 and 65 cm and biomasses between 45 and 190 g DW (Tab. 3.1 on the following page). Biomasses in mid-season increased in the order *Onobrychis*, *Lotus*, and *Cichorium* (intercepts of the multiple linear regression models; Tab. 3.2 on page 62, Fig. 3.1 on page 61). There were no significant differences between the biomasses of the two cultivars of *Onobrychis* (41 g; P=0.516) or between the cultivars of *Lotus* (56 g; P=0.299; Tab. 3.2 on page 62). However, in *Cichorium*, biomasses in mid-season were significantly higher in Lacerta (119 g) than in Puna (119-42=77 g; $P<0.01$; Tab. 3.2 on page 62). This and the lack of fit of the regression model for *Cichorium* (Fig. 3.1 on page 61) was due to the fact that from the 9^{th} week after sowing, some individual plants of the cultivar Lacerta started to produce a large amount of stems, whereas those of the cultivar Puna did not.

Table 3.1: *Height, specific leaf area (SLA), leaf mass fraction (LMF), stem mass fraction (SMF) and root mass fraction (RMF) of the entire plant at the beginning (4^{th} week) and at the end (20^{th} week) of the experiment. Shown are means and standard errors of the mean.*

		Onobrychis viciifolia		***Lotus corniculatus***		***Cichorium intybus***	
		Visnovsky	commercial seed	Lotar	Oberhaunst.	Lacerta	Puna
Height (cm)	4^{th} week	10.5±1.0	12.2±1.2	11.2±1.0	8.3±1.3	11.9±1.3	11.8±1.0
	20^{th} week	40.4±3.4	44.0±1.5	51.73±3.4	61.8±3.1	63.2±7.8	47.2±5.1
SLA (m^2 kg^{-1})	4^{th} week	13.1±1.2	13.1±0.7	20.9±1.4	22.4±0.6	26.4±1.6	27.3±0.6
	20^{th} week	12.1±0.8	10.5±0.6	22.0±1.0	20.8±4.5	10.2±3.5	16.0±1.1
LMF (% DM)	4^{th} week	84.9±2.4	85.4±2.9	63.1±0.7	64.5±0.8	84.8±1.0	86.1±0.2
	20^{th} week	28.5±5.4	31.7±4.0	16.0±1.3	20.8±1.4	43.2±8.3	35.0±3.3
SMF (% DM)	4^{th} week	4.4±2.3	5.2±2.6	30.29±0.7	28.3±0.8	0.5±0.5	0.7±0.4
	20^{th} week	44.9±2.5	38.5±2.0	52.79±3.6	55.5±2.9	12.8±10.4	3.3±0.5
RMF (% DM)	4^{th} week	10.8±0.1	9.4±0.6	6.6±0.1	7.2±0.2	14.7±1.1	13.2±0.5
	20^{th} week	26.5±3.1	29.7±2.3	31.21±2.4	23.8±1.6	44.0±7.0	61.7±3.1

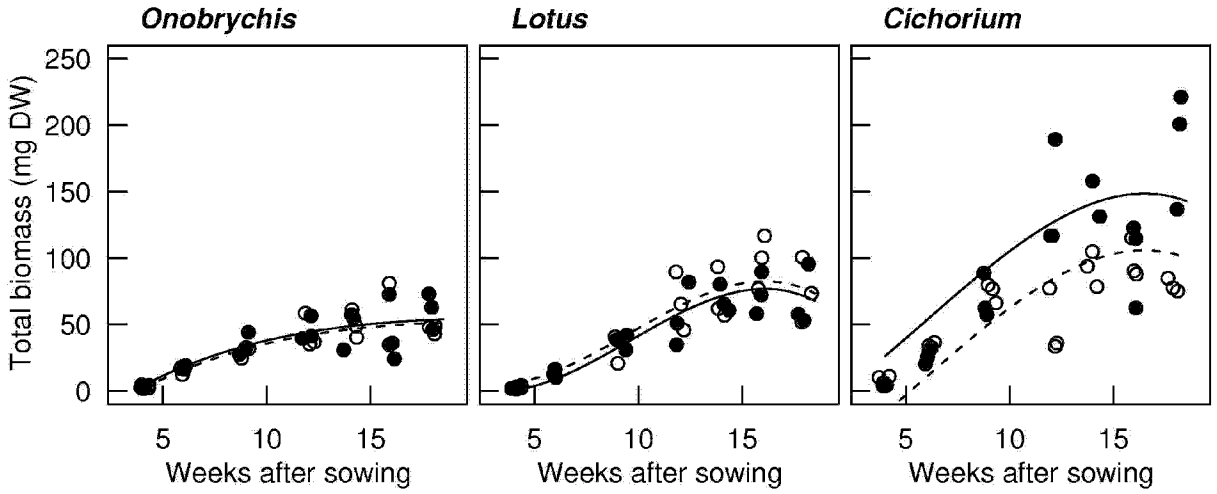

Figure 3.1: *Total biomass per pot in the course of the experiment (growth). Data are represented as dots, the linear models as lines, for statistical details see Tab 3.2 on the following page. Left:* Onobrychis*; commercial seed (open symbols, dashed line) and Visnovsky (closed symbols, solid line). Middle:* Lotus*; Oberhaunstädter (open symbols, dashed line) and Lotar (closed symbols, solid line). Right:* Cichorium*; Puna (open symbols, dashed line) and Lacerta (closed symbols, solid line).*

3.4.2 Leaf development

Comparing the first to the last harvest, specific leaf areas (SLA) decreased only marginally and non-significantly in *Onobrychis* (from 13.1 to 11.3 m^2 kg^{-1}, t-test: P=0.056) and *Lotus* (from 21.7 to 21.4 m^2 kg^{-1}, P=0.909), but strongly in *Cichorium* (across cultivars from 26.9 to 13.1 m^2 kg^{-1}, P$<$0.001; Tab. 3.1 on the facing page), respectively. In *Onobrychis* and *Lotus*, the individual leaves expanded rapidly to full size; hence, the majority of the leaves on the plants of these two species were mature and fully expanded at all times, explaining the almost constant average SLA per pot during the experiment. In contrast, the massive expansion of leaves of *Cichorium* affected all leaves on a given plant and extended over the entire experiment, resulting in a decreasing average SLA per pot during the experiment.

3.4.3 Biomass allocation to leaves, stems and roots

In all three species and in all six cultivars, the relative contributions of stems (SMF) and roots (RMF) to the total biomasses of the plants increased, while the relative contributions of leaves decreased during plant development (Tab. 3.1 on the preceding page).

Table 3.2: *Total biomass in the course of the experiment (growth). Estimated coefficients and p-values of multiple linear regression models presented in Eqn. 3.1 on page 57, for each species. Effect of time P<0.001 for all species (partial F-tests between the full and the reduced model without β_2, β_3, β_4).*

		Onobrychis		***Lotus***		***Cichorium***	
		Estimate	p-value	Estimate	p-value	Estimate	p-value
Intercept:	β_0	2.278	***	52.695	***	119.29	***
	$c \cdot \beta_1$	-2.33	0.516	5.696	0.299	-42.66	**
Temporal:	$t \cdot \beta_2$	2.992	**	8.385	0.392	10.137	*
	$t^2 \cdot \beta_3$	-0.251	*	-0.356	0.055	-0.657	0.098
	$t^3 \cdot \beta_4$	0.008	0.749	-0.072	***	-0.041	0.694

Code of the levels of significance: p $<$ 0.05 *, p $<$ 0.01 **, p $<$ 0.001 ***

In the course of the experiment, the leaf fractions of the harvestable biomasses (LMFH) decreased from 100, 79, 100 % to 61, 32 and 91 % in *Onobrychis*, *Lotus* and *Cichorium* respectively (Fig. 3.2 on the facing page). In an early phase, this was due to the formation of stems and later, after reaching a plateau at maturity, due to the loss of leaves at the onset of senescence. The leaf fractions of the harvest were well represented by polynomials of 3^{rd} degree for *Onobrychis* and *Lotus*. However, in *Cichorium*, the leaf fraction of the harvest could not be modelled satisfyingly because plants grew very heterogeneously and stems were produced by a few individual plants only. With regard to the leaf fraction of the harvest, no significant differences were found between the cultivars of *Onobrychis* (P=0.233; Tab. 3.3 on the next page) or the cultivars of *Lotus* (P=0.299). Differences between the two cultivars of *Cichorium* are obvious as stems were only produced by the cultivar Lacerta.

3.4.4 Tannin concentrations in leaves, stems and roots

Across species, tannin concentrations in both leaves and stems were highest in *Onobrychis*, intermediate in *Lotus* and lowest in *Cichorium* (intercepts of multiple linear regression models; Tab. 3.4 on page 66). Averaged over the entire experiment, tannin concentrations in leaves were higher than those in stems in *Onobrychis* (wilcoxon rank sum test; P<0.001) and in *Lotus* (P<0.001) but equally low in *Cichorium* (P=0.098). While roots of *Onobrychis* (4 mg g^{-1} DW) and *Cichorium* (2 mg g^{-1} DW) were almost

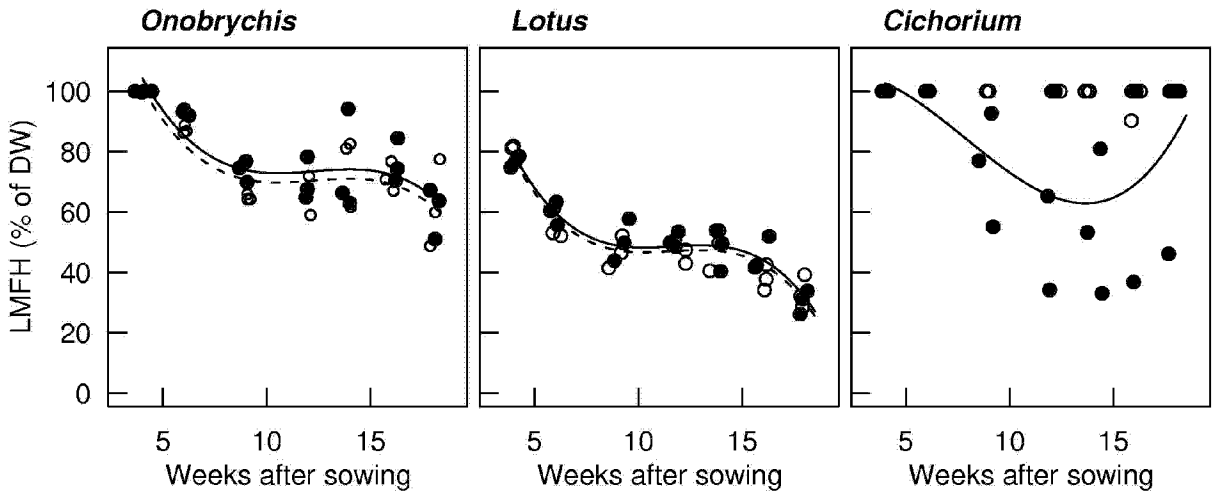

Figure 3.2: *Leaf mass fraction of the harvestable biomass (> 5cm aboveground; LMFH) in the course of the experiment. Data are represented as dots, the linear regression models as lines, for statistical details see Tab 3.3. Left:* Onobrychis*; commercial seed (open symbol, dashed line) and Visnovsky (closed symbol, solid line). Middle:* Lotus*; Oberhaunstädter (open symbol, dashed line) and Lotar (closed symbol, solid line). Right:* Cichorium*; Puna (open symbols) which did not produce stems during the entire experiment and Lacerta (closed symbols, solid line).*

Table 3.3: *Leaf fraction of harvestable biomass (> 5 cm). Estimated coefficients and p-values of multiple linear regression models presented in Eqn. 3.2 on page 58, for each species. For Cichorium, the term λ_1 has been omitted as the cultivar Puna did not produce stems during the experiment. Effect of time: P<0.001 for* Onobrychis *and* Lotus*, but P=0.091 for* Cichorium *(partial F-tests between the full and the reduced model without λ_2, λ_3, λ_4).*

		Onobrychis		***Lotus***		***Cichorium***	
		Estimate	p-value	Estimate	p-value	Estimate	p-value
Intercept:	λ_0	72.97	***	48.41	***	67.5	***
	$c \cdot \lambda_1$	-3.118	0.223	-1.573	0.299	–	–
Temporal:	$t \cdot \lambda_2$	0.372	0.62	0.383	0.392	-3.626	0.246
	$t^2 \cdot \lambda_3$	0.186	*	0.081	0.055	0.571	0.056
	$t^3 \cdot \lambda_4$	-0.063	**	-0.075	***	0.056	0.462

Code of the levels of significance: p $<$ 0.05 *, p $<$ 0.01 **, p $<$ 0.001 ***

devoid of tannins, roots of *Lotus* had relatively high tannin concentrations (26 mg g^{-1} DW, compared to only 12 mg g^{-1} DW in stems and about 43 mg g^{-1} DW in leaves; Tab. 3.4 on page 66). For a given plant organ, the only significant difference between cultivars was found in the leaves of *Lotus*, where cultivar Oberhaunstädter had higher tannin concentrations in mid-season (47 mg g^{-1} DW) than cultivar Lotar (38 mg g^{-1} DW; $P<0.01$; Tab. 3.4 on page 66).

3.4.5 Dynamics of tannin concentrations over time

In the course of the experiment, significant changes of tannin concentrations were only found in leaves (Fig. 3.3 on the facing page; Tab. 3.4 on page 66) but not in stems or roots (Tab. 3.4 on page 66). During the experiment, the tannin concentration in leaves increased strongly and approximately linearly in *Onobrychis* (from 52 to 86 mg g^{-1} DW; $P<0.001$) and in *Lotus* (Oberhaunstädter: from 26 to 59 mg g^{-1} DW; Lotar: from 23 to 49 mg g^{-1} DW; $P<0.001$) but not in *Cichorium* ($P=0.527$), which was the only species with a strongly decreasing SLA (Tab. 3.1 on page 60). Between cultivars there were no significant differences in slopes for any of the species.

Despite the fact that tannin concentrations approximately doubled in leaves of *Onobrychis* and *Lotus* during the experiment, there was no statistical evidence for a change in tannin concentrations in the harvestable biomasses in any of the investigated species or cultivars (*Onobrychis*: 62, *Lotus*: 26, *Cichorium*: 5 mg g^{-1} DW; Fig. 3.3 on the next page; Tab. 3.4 on page 66). With regard to tannin concentration in harvestable biomass, the effect of the increasing tannin concentration in leaves was evened out by dilution due to the increasing proportion of 'tannin-poor' stems.

Tannin concentrations on the level of the entire plant increased significantly in *Lotus* but decreased non-significantly in *Onobrychis* and *Cichorium* (Fig. 3.3 on the next page, Tab. 3.4 on page 66). These results reflect the fact that all investigated species invested an increasingly large proportion of their acquired biomass in the production of roots (RMF; Tab. 3.1 on page 60); but roots of *Lotus* contained high amounts of tannins, whereas roots of *Onobrychis* and *Cichorium* did not (Tab. 3.4 on page 66).

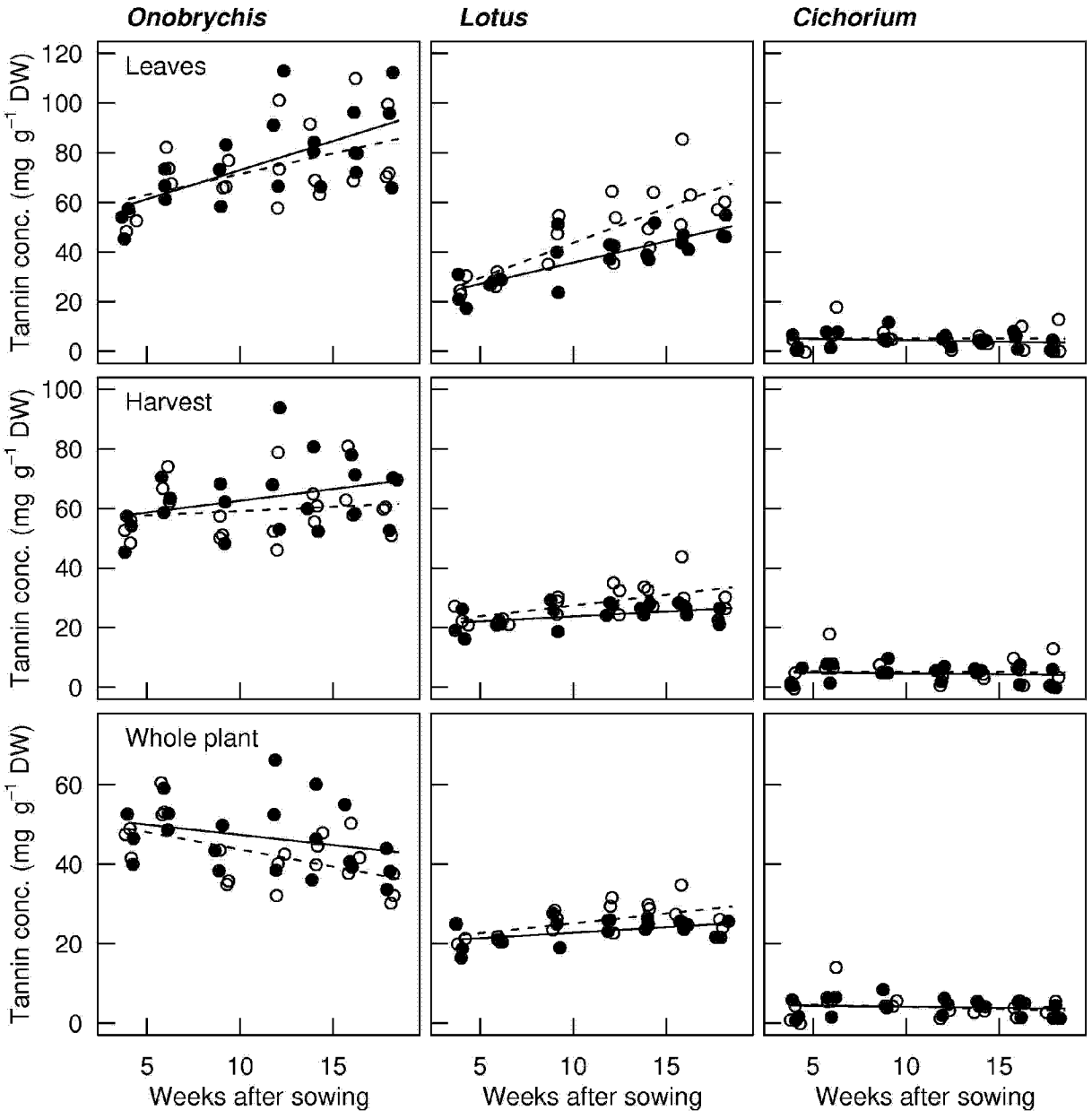

Figure 3.3: *Tannin concentrations in the course of the experiment in (a) leaves, (b) harvestable biomass and (c) in the entire plant. Data are represented as dots, the linear regression models as lines, for statistics see Tab. 3.4 on the next page. Left:* Onobrychis*; commercial seed (open symbol, dashed line) and Visnovsky (closed symbol, solid line). Middle:* Lotus*; Oberhaunstädter (open symbol, dashed line) and Lotar (closed symbol, solid line). Right:* Cichorium*; Puna (open symbol, dashed line) and Lacerta (closed symbol, solid line).*

Table 3.4: *Tannin concentrations in leaves, stems, roots, in harvestable biomass and in the entire plant. Estimated coefficients and p-values of the multiple linear regression models presented in Eqn. 3.3 on page 58, for each species. Intercepts can be interpreted as the tannin concentration in mid-season, the sum of the temporal coefficient as the weekly change in tannin concentration (mg CT g^{-1} DW $week^{-1}$).*

		Onobrychis		***Lotus***		***Cichorium***	
		Estimate	p-value	Estimate	p-value	Estimate	p-value
Leaves							
Intercept:	τ_0	75.99	***	37.91	***	4.29	***
	$c \cdot \tau_1$	-2.46	0.579	9.34	**	0.82	0.496
Temporal:	$t \cdot \tau_2$	2.33	***	1.72	***	-0.11	0.527
	$t \cdot c \cdot \tau_3$	-0.67	0.469	1.09	0.061	0.099	0.695
Stems							
Intercept:	τ_0	24.88	***	12.03	***	7.8	**
	$c \cdot \tau_1$	-2.21	0.591	0.81	0.563	–	–
Temporal:	$t \cdot \tau_2$	-1.25	0.131	-0.19	0.376	-0.37	0.51
	$t \cdot c \cdot \tau_3$	0.69	0.489	0.42	0.156	–	–
Roots							
Intercept:	τ_0	3.13	***	27.35	***	2.04	*
	$c \cdot \tau_1$	1.74	0.64	-2.97	0.491	0.88	0.453
Temporal:	$t \cdot \tau_2$	-0.29	0.056	0.53	0.404	-0.07	0.69
	$t \cdot c \cdot \tau_3$	-0.14	0.482	0.39	0.681	0.09	0.734
Harvest							
Intercept:	τ_0	63.58	***	24.16	***	4.57	***
	$c \cdot \tau_1$	-4.1	0.216	4.16	**	0.53	0.656
Temporal:	$t \cdot \tau_2$	0.79	0.11	0.33	0.083	-0.06	0.716
	$t \cdot c \cdot \tau_3$	-0.5	0.467	0.39	0.15	0.05	0.85
Whole plant							
Intercept:	τ_0	46.72	***	23.12	***	4.05	***
	$c \cdot \tau_1$	-4.12	0.093	2.64	**	-0.11	0.882
Temporal:	$t \cdot \tau_2$	-0.51	0.154	0.28	*	-0.05	0.652
	$t \cdot c \cdot \tau_3$	-0.36	0.48	0.21	0.292	-0.05	0.761

Code of the levels of significance: p $<$ 0.05 *, p $<$ 0.01 **, p $<$ 0.001 ***

3.5 Discussion

We found large and significant differences between the tannin concentrations in leaves, stems and roots. Where comparable the CT concentrations found here were in the range of plant CT concentrations previously reported for these species (Terrill et al., 1992; Jackson et al., 1996; Joseph et al., 1998; Gebrehiwot et al., 2002; Wen et al., 2003). As plants developed, the relative biomass proportions of leaves decreased and the proportions of stems and roots increased consistently in all investigated plant species and cultivars. Given that both, variations in tannin concentrations between different plant organs (e.g. Gebrehiwot et al., 2002) and shifting proportions between them (e.g. Borreani et al., 2003) can be found in many plant species, it is surprising that this knowledge has never been used before to model and predict CT dynamics in harvestable biomass of developing plants.

3.5.1 Modelling the seasonal dynamics of tannin concentrations in harvestable biomass

As suggested in the introduction, the temporal dynamics of the tannin concentration in harvestable biomass (T_{harvest}) can be written as a function of the proportion of leaves and stems in the harvested biomass over time and of the temporal dynamics of the CT concentration within these organs:

$$T_{\text{harvest}}(c,t) = L(t) \cdot T_{\text{leaves}}(t) + [1 - L(t)] \cdot T_{\text{Stems}}(t) \quad \ldots \quad (3.4)$$

By using the least square estimates presented in table 3.4 on the preceding page, equation 3.4 gives a continuous and quantitative estimate of the tannin concentrations in the harvestable biomass for any of the investigated species and cultivars. The function $L(t)$ is, in our case, a polynomial of 3^{rd} degree (Tab. 3.3 on page 63; Fig. 3.2 on page 63) and models the proportion of leaves and stems in the harvest over time. $T_{\text{leaves}}(t)$ and $T_{\text{stems}}(t)$ are a linear function of time and a constant respectively, and model the dynamics of the CT concentrations within leaves and stems. It may be seen from this equation that biomass allocation itself can be an active driver of the tannin concentration in the harvestable biomass, provided that (i) the difference in tannin concentration between leaves and stems is relatively large and (ii) the proportion between these organs changes over time. In our experiment, this was clearly the case in *Onobrychis*

and *Lotus*. With regard to tannin concentrations in the harvest of these species, we found that the strongly increasing tannin concentrations in leaves were almost exactly evened out by dilution from an increasingly large proportion of 'tannin-poor' stems in the harvestable biomass.

3.5.2 Differences in biomass allocation may explain conflicting results

Here, the tannin concentrations in the harvestable biomasses were more or less stable during the season in all investigated species. However, Wen et al. (2003) reported for *Lotus* that tannin concentrations declined in spring after the onset of grazing in the first experimental year and were stable in the second year. In contrast, Gebrehiwot et al. (2002) and Roberts et al. (1993) found similar tannin concentrations in spring and summer but lower concentrations in autumn. Unfortunately, none of these studies reported the relative contribution of leaves and stems to biomass at the time of harvest. Nevertheless, both the decline of tannin concentrations after the onset of grazing and the lower concentrations in autumn could have resulted from a decreasing proportion of leaves in the harvestable biomass due to selective grazing or due to the onset of senescence, respectively.

A study on *Onobrychis viciifolia* (i.e. Borreani et al., 2003) is the only investigation known to us that reported CT concentrations in the harvestable biomass and biomass allocation to both leaves and stems for one of the species analysed in our study. From a diagram in that report it can be estimated that LMFH was 90 % in the first and 22 % in the last harvest (seven weeks later). Our model (Eqn. 3.4 on the previous page), using the least square estimates for the dynamics of CT concentrations in leaves and stems of *Onobrychis* (Tab. 3.4 on page 66) and the LMFH values reported in Borreani et al. (2003), predicts CT concentrations in the harvestable biomass to decrease by 40.3 or 44.4 % (models for cv Visnovsky or commercial seed) during the season compared to the actually observed decrement of 40 % (absolute values are not comparable because of the use of different reference tannins). Hence, the decreasing CT concentrations in the harvestable biomass of *Onobrychis* reported in Borreani et al. (2003) are consistent with the stable CT concentration in our experiment if one accounts for the different dynamics of biomass proportions between leaves and stems.

3.5.3 Tannin synthesis and dilution by growth co-occur

In leaves of *Onobrychis* and *Lotus*, where the bulk mass of leaves was mature and the mean SLA per pot approximately constant, we found significant increments of the tannin concentrations during the season. Such an accumulation of tannins in already expanded leaves is in agreement with outdoor studies on several other plant species (Feeny, 1970; Parker, 1977; Glyphis & Puttick, 1988; Riipi et al., 2002). In contrast, in the strongly growing leaves of *Cichorium*, tannin concentrations were stable over time. This suggests that a continuous production of tannins was matched by a concurrent accumulation of non-tanniferous plant material (growth and storage). Similarly, Joseph et al. (1998), who investigated tannin concentrations in the expanding leaves of *Onobrychis viciifolia* from the earliest developmental stages to the maturation of the leaves, reported that despite of a strong increment in the absolute amount of tannins per leaf, the tannin concentration of these leaves tended to decrease until maturation. These results hint that whenever growth and storage play important roles, the interpretation of concentrations alone can be ambiguous because the effect of tannin synthesis and dilution from growth and storage are confounded. Dilution by growth and storage are sometimes used as a post-hoc explanation for unexpected results. However, they are rarely acknowledged as an active driving force of tannin concentration dynamics. We suggest that for the prediction and modelling of seasonal and developmental variations of tannin concentrations, dilution processes should receive as much attention as tannin synthesis and its regulation.

3.5.4 Dynamics of tannin concentrations in different plant parts need not be correlated

We found that tannin concentrations can increase in leaves, be stable in the harvestable biomass and yet, decrease on the level of the entire plant during plant development, as shown in *Onobrychis*. The different levels and dynamics of tannin concentrations found in different plant parts challenge the common assumption that for a given plant, a 'level of defence' exists (e.g. Stamp, 2003). In fact, different herbivores or pathogens may not only find very different concentrations of tannins but also different dynamics of tannin concentrations in their diets during plant development depending on the plant parts they consume.

In ecology, concentration data of plant parts, in particular of leaves, are often used in

order to assess plant biomass allocation to defence (Koricheva, 1999). This practice relies heavily on the assumption that concentrations of allelochemicals in leaves correlate with the relative biomass investment expended by the entire plant. However, in our experiment there was no general agreement between the tannin concentrations in leaves and the proportion of the net acquired biomass invested in the production of tannins. Concentration data of plant parts do therefore not allow to elucidate the efforts expended by the whole plant, and thus are not adequate by themselves for the discussion of the resource trade-offs between defence and growth (Koricheva, 1999; Kurokawa et al., 2004). We found no conclusive evidence that periods of strong growth are correlated with low defence allocation as suggested by the GDB hypothesis (Herms & Mattson, 1992; Lerdau et al., 1994). For example in *Onobrychis*, relative growth rates and the relative biomass investment in tannins were both maximal at the beginning of the experiment and decreased thereafter. This was due to the fact that in an early developmental phase, a large proportion of the net acquired biomass was invested in the production of tannin-rich leaves, whereas later on, increasingly large proportions of biomass were invested in tannin-poor stems and roots. Thus, changes in the proportion of the net acquired biomass invested in tannins (tannin concentrations in the entire plants), reflect changing proportions of plant organs with different CT concentrations rather than different defence strategies.

3.5.5 Conclusions

In developing plants, tannin concentrations are a result of antagonistic effects between tannin synthesis and dilution by accumulation of non-tanniferous material. Therefore, models to predict tannin concentrations, in particular in strongly growing plants and/or in very heterogeneous plant material, should not only be based on knowledge of tannin synthesis but also consider dilution processes. For the practical application in agronomy, it may be expected that tannin concentrations in the harvestable biomass of mixed stands are functions of the biomass proportions between tanniferous and non-tanniferous plant species - and in pure stands, of the biomass proportion between tannin-rich leaves and tannin-poor stems in the harvest. For the breeding of tanniferous forage crops, it seems promising to select persistent plants with high leaf:stem ratios.

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Chapter 4

Tanniferous forage plants: agronomic performance, palatability and efficacy against parasitic nematodes in sheep

HÄRING D.A.[12], SCHARENBERG A.[34], HECKENDORN F.[45], DOHME F.[3], LÜSCHER A.[1], MAURER V.[5], SUTER D.[1], HERTZBERG H.[6]

Invited article: submitted to *Renewable Agriculture and Food Systems*.

This chapter links up with work done by the other modules of the *Tannin-Project*, namely with the module *Animal Nutrition* and the module *Parasitology*. It presents original data of a three year field experiment of the cultivation of tanniferous forage plants, compares the palatability of various tanniferous forage plants to a grass / legume mixture and finally summarizes the antiparasitic activity of conserved sainfoin hay and silage against gastrointestinal parasites in sheep. The author of this thesis contributed to the current chapter by providing the data and the text concerning a three-year field experiment with 12 cultivars of 4 tanniferous plant species, by writing the conference article that led to the invitation to write a full research paper for *Renewable Agriculture and Food Systems* from data and text contributions of all three modules of the *Tannin-Project* and by organizing and writing the current chapter form drafts of all three modules.

[1]Agroscope Reckenholz-Tänikon Research Station ART, Zurich
[2]Institute of Plant Sciences, ETH Zurich
[3]Agroscope Liebefeld-Posieux Research Station ALP, Posieux
[4]Institute of Animal Sciences, ETH Zurich
[5]Research Institute of Organic Agriculture, Frick
[6]Institute of Parasitology, University of Zurich

4.1 Abstract

Tanniferous forage plants can have beneficial effects on ruminant productivity and health (improved protein supply, bloat safety and antiparasitic properties). However, condensed tannins can also lower palatability, voluntary feed intake and digestibility. The aim of our interdisciplinary project was to generate basic knowledge on plant management, feed palatability and the antiparasitic properties of tanniferous forage plants for their practical application in agronomy focussing on their usefulness in controlling gastrointestinal nematodes in organic farming. We found that *Onobrychis viciifolia* (sainfoin), *Lotus corniculatus* (birdsfoot trefoil) and *Cichorium intybus* (chicory) were suitable for cultivation under the given temperate climatic conditions whereas *Lotus pedunculatus* (big trefoil) was soon outcompeted by unsown species. Growing the tanniferous plant species in a mixture with *Festuca pratensis* (meadow fescue) rather than in a monoculture had the advantage of increasing total dry matter yield (especially in the case of tanniferous legumes) and of reducing the dry matter proportions of unsown species. However, due to dilution by non-tanniferous *F. pratensis*, the tannin concentrations of mixtures were clearly lower and the seasonal fluctuations in tannin concentrations greater than that of monocultures. Across species, tannin concentrations were highest for *O. viciifolia*, followed by *L. corniculatus* and very low for *C. intybus*. Palatability of all tanniferous forages was comparable to that of a ryegrass/clover mixture when fed as dried forage and, when offered as silage, palatability of *O. viciifolia* was clearly superior to that of the respective ryegrass/clover control. Administration of dried or ensiled *O. viciifolia* reduced parasite egg counts in faeces of lambs co-infected with the gastrointestinal nematode species *Haemonchus contortus* and *Cooperia curticei*. We conclude that *O. viciifolia* is the most promising among the tested tanniferous forage plant species due to its suitability for cultivation, its high tannin concentration, its high palatability and its antiparasitic activity even in dried or ensiled form.

4.2 Introduction

Recent experiments in agronomy and parasitology suggest that moderate dietary concentrations of condensed tannins can affect the health and performance of sheep and other small ruminants beneficially (Aerts et al., 1999; Barry & Mcnabb, 1999; Min et al., 2003; Hoste et al., 2006; Mueller-Harvey, 2006). For example, condensed tannins were found to increase life weight gain, fecundity and wool production in sheep (Min et al., 2001; Ramirez-Restrepo et al., 2004). With regard to animal health, condensed tan-

nins are known to diminish the risk of bloat, lower faecal worm egg excretion and reduce worm burdens of parasitized ruminants (Hoste et al., 2006; Mueller-Harvey, 2006). However, high concentrations of condensed tannins can have a negative impact on ruminants. They can lower palatability and digestibility (Titus et al., 2000; Mueller-Harvey, 2006) and, in extreme cases, they can lead to mucosa lesions (Provenza et al., 2003).

In an interdisciplinary project involving research on plant cultivation, animal nutrition and parasitology, we addressed key questions concerning the implementation of tanniferous forage plants as an alternative control strategy against gastrointestinal nematodes in animal husbandry: Which tanniferous species and cultivars perform well with regard to agronomic properties such as yield, persistence and competitive ability? What are their concentrations of condensed tannins and what are the most important drivers of fluctuations in tannin concentrations under field conditions? How palatable are different tanniferous forage plant species in comparison to a ryegrass/clover mixture? How effective is the most promising plant species against gastrointestinal nematodes in sheep? Can tanniferous forages be conserved without losing their desirable anthelmintic properties?

4.2.1 Plant sciences and forage cultivation

The expressions 'condensed tannins' or synonymously 'proanthocyanidins' summarize a large and chemically diverse group of phenolic polymers with the ability to bind proteins and other macromolecules. Apart from their strong affinity for proteins, condensed tannins can form complexes with metal ions and have radical scavenging properties (Haslam, 1996). The structure of condensed tannins consists of a linkage of a series of monomers based on flavan-3-ol nuclei or derivatives thereof (Waterman & Mole, 1994). Variations in the structure of condensed tannins (and supposedly of their bioactivity) can occur through differences in the number of monomers, the positions between which these monomers are interlinked, the oxygenation pattern of the monomers or the stereochemistry of the polymer (Waterman & Mole, 1994; Marles et al., 2003). Condensed tannins exist as water soluble oligomers and as insoluble polymers (Haslam, 1996). Tanniferous plants usually contain mixtures of differently structured tannins rather than one specific type of molecule (Koupai-Abyazani et al., 1993; Marles et al., 2003). The quantity and structural composition of tannins vary between different tissues and can change during plant development (Koupai-Abyazani et al., 1993; Häring et al., 2007). Condensed tannins occur predominantly in woody plants but can also be found

in some herbaceous plant species, for example in representatives of the Rosaceae and Fabaceae families (Waterman & Mole, 1994). Within plants, condensed tannins are concentrated in cell vacuoles (Marles et al., 2003) and usually occur in higher concentrations in leaves and reproductive organs than in stems and roots (e.g. Häring et al., 2007). The bioactivity of condensed tannins is thought to be a function of tannin concentrations defined as the ratio between consumed tannin and consumed protein or feed (Waterman & Mole, 1994) rather than of the absolute amount of consumed tannin. As a rule of thumb it has repeatedly been suggested that tannin concentrations below 50 g kg^{-1} DM produce positive effects in ruminants while concentrations in excess of 50 g kg^{-1} DM affect the animal negatively (Aerts et al., 1999; Min et al., 2003). Today's experimental evidence relativises this rule to the extent that tannins from different sources may vary in their 'potency' (Mueller-Harvey, 2006) and that it is likely that different thresholds need to be defined for different tannins and, thus, different forage plant species.

Genotypic and phenotypic variation of type and quantity of secondary metabolites have often been studied within the paradigm that their primary role is plant defensive and their effect on consumers detrimental. The accumulated experimental evidence for and against the so called plant defence theory (Bryant et al., 1983; Coley et al., 1985; Herms & Mattson, 1992; Stamp, 2003) is equivocal (Koricheva et al., 1998; Koricheva, 2002) and its usefulness for the practical application in agronomy disputable (Häring et al., 2007). Previous studies of seasonal fluctuations of tannin concentrations in forage plants yielded conflicting results (Roberts et al., 1993; Gebrehiwot et al., 2002; Borreani et al., 2003; Wen et al., 2003; Häring et al., 2007), and mechanisms driving seasonal and developmental dynamics of tannin concentrations under field conditions are not yet well understood. Based on an experiment with potted *O. viciifolia* and *L. corniculatus*, it has been suggested that fluctuations in condensed tannin concentrations in harvestable aboveground biomass are functions of biomass proportions between tannin-rich leaves and tannin-poor stems in pure stands and of biomass proportions between tanniferous and non-tanniferous plant species in mixed stands (Häring et al., 2007). At present, it is unclear to what extent these hypotheses hold true under field conditions. For a successful implementation of a gastrointestinal nematode control strategy based on tanniferous forage plants, it is essential that the tanniferous forage crop provides a high yield, has a high competitive ability and an elevated but predictable concentration of condensed tannins. Especially under low input conditions, the agronomic performances of species mixtures have been reported superior to those of monocultures. In comparison to monocultures, mixtures are often characterised by higher dry matter

yields (e.g. Kirvan et al., 2007), improved evenness of the seasonal growth patterns (Elgersma et al., 1998) and increased resistance against weed invasion (Kirvan et al., 2007). Until now, comparative assessments of agronomic performances of promising tanniferous forage plants and concurrent investigations of tannin concentration dynamics under field conditions have been scarce.

Therefore we evaluated yield, resistance to weed invasion and tannin concentrations of 12 cultivars of four commercially available, tanniferous forage plant species which were either sown in a monoculture or in a mixture with the grass species *Festuca pratensis* in a three-year field experiment. We put special emphasis on investigating to what extent potential seasonal fluctuations of condensed tannin concentrations in the yield can be attributed to shifts in the relative dry matter contribution of tanniferous plants to total dry matter yield.

4.2.2 Animal nutrition

Palatability designates the sum of all physical and chemical characteristics of a diet that evoke appetite such as olfactory, gustatory and tactile stimuli during foraging and chewing (Baumont, 1996). Repeated feeding of a particular diet enables an animal to anticipate potential nutritional and physiological postingestive feedbacks and eventually to develop a preference for or an aversion against it (Provenza, 1995). The perception of a diet, and subsequent feed preferences, vary among individual animals according to differences in physiology and experience (Scott & Provenza, 1999). Therefore, palatability and food preference are questions of physiological traits of the animal, of individual experience and of the feed quality of the forage in question in relation to the range of feeds available to the animal. There is a wealth of evidence suggesting that condensed tannins in plants influence the voluntary feed intake, palatability and digestibility of the fodder (Barry & Mcnabb, 1999; Min et al., 2003; Mueller-Harvey, 2006). It is thought that the importance of condensed tannins in animal nutrition lies mainly in their ability to bind proteins. For example, the astringent taste of tanniferous plants is a consequence of the binding of tannins to salivary proteins. The lower true digestibility of feed proteins, the increase of the excretion of endogenous protein in pigs (Jansman et al., 1995), and the lower ruminal protein degradability combined with a lower apparent digestibility in ruminants (Waghorn et al., 1994b) are results of the binding of tannins to feed protein and endogenous proteins. While protein precipitation in the intestine lowers amino acid availability and affects the ruminant adversely, protein precipitation and protein protec-

tion from microbial degradation in the rumen can be desirable and beneficial to the animal (Min et al., 2005). The binding of condensed tannins to protein in the rumen (pH $6 - 7$) lowers ammonia production and reduces the metabolic strain of the liver (Barry & Mcnabb, 1999). Furthermore, it enhances the protein flow towards the small intestine and, provided the tannin-protein-complexes dissociate in the more acid medium of the abomasum (pH < 3), raises the animal's supply of essential amino acids (McNabb et al., 1993; Mueller-Harvey, 2006). An improved protein supply of the ruminant is also one of the possible pathways leading to an increased tolerance of ruminants to gastrointestinal parasite infections (Coop & Holmes, 1996; Hoste et al., 2006). In addition to the likely impact of condensed tannins on nitrogen metabolism, tannins may also affect the mineral supply of the animal (Freeland et al., 1985; Waghorn et al., 1994a). For the practical application of tanniferous forages in livestock production, e.g. as an alternative nematode control strategy, administration of forage plants in a conserved form (either as dried or ensiled forage) is of great interest. In previous experiments, condensed tannins were reported to depress proteolysis thereby reducing the loss of forage protein during the ensiling process (Salawu et al., 1999). Furthermore, it was observed that ensiling reduced tannin concentrations of sorghum (Ott et al., 2005). Up to now, little is known about the effect of ensiling on the palatability of tanniferous forage plants.

Feeding trials provide an ideal means of obtaining a brief and summary-like answer to the complex question of whether or not a certain (tanniferous) forage plant species is suitable for its consumer and allow an assessment of voluntary feed intake and possible side-effects of the feed. In this study, we aimed to identify the most promising tannin containing plant species and conservation method in terms of the wethers' acceptance and, thus, palatability of dried or ensiled *O. viciifolia*, *L. corniculatus*, and *C. intybus* in relation to a dried or ensiled non-tannin containing ryegrass/clover mixture, respectively.

4.2.3 Parasitology

Infections with gastrointestinal nematodes represent a major constraint on the profitability of sheep, goat and cattle production. This is particularly pronounced in low-input farming where animals are grazing on pastures in contrast to intensive production systems in which access to pasture is limited or even absent (Waller & Thamsborg, 2004). The organic farmer is confronted with the problem of (i) reducing the infection pressure to an acceptable level and (ii) having largely to resign himself to the use of

conventional anthelmintic drugs in order to keep within the organic guidelines. As complementary approaches to control gastrointestinal parasitism of small ruminants like the exploitation of the genetic resistance of livestock (e.g. Woolaston & Baker, 1996; Woolaston & Piper, 1996), biological control of the free living larval stages in the faeces by means of nematophagous fungi (Larsen, 1999; Eysker et al., 2006), manipulation of grazing management (Niezen et al., 1996) and dietary supplementation with protein (Coop & Kyriazakis, 2001) were each only partially effective, the control of endoparasites remains largely based on the application of synthetic anthelmintic drugs. The development of gastrointestinal parasite populations resistant against most of the currently available anthelmintics further highlights the need for alternative parasite control strategies (Waller, 1997). Recent experiments with tanniferous forages administred to sheep and goats infected with gastrointestinal nematodes yielded promising results (Hoste et al., 2006). Tanniferous forages that were grazed, or freshly administered, to naturally or artificially infected animals often resulted in a decreased faecal egg count (i.e. the number of worm eggs per gram faeces of the host) which was sometimes accompanied by a reduction of worm burden (i.e. the number of adult worms) or reductions in worm fecundity. The potential of condensed tannins as an antiparasitic agent has been substantiated by various in-vitro and in-vivo experiments: Condensed tannins were found to reduce the parasites' motility, possibly by binding to surface proteins, to exert a toxic effect on parasite eggs, larvae or adult worms and to hinder larval development (Athanasiadou et al., 2001; Molan et al., 2003; Hoste et al., 2006). Alternatively or complementary to these 'direct effects', condensed tannins can exert indirect effects. For example, they may help to improve the host's immune response to parasitism (i.e. resistance) by enhancing the ruminant's supply of proteins and essential amino acids (Hoste et al., 2006).

Administering tanniferous forages to parasitized ruminants in a conserved form, either as hay or silage, could potentially enhance the practicability and efficacy of the treatment. However, experiments with conserved forages remain few. Therefore, we tested the feeding of *Onobrychis viciifolia* hay and silage, respectively, to lambs co-infected with *Haemonchus contortus* and *Cooperia curticei* in comparison to non-tanniferous control feeds (Heckendorn et al., 2006).

4.3 Material & Methods

4.3.1 Agronomic performance of tanniferous forage plants

We assessed the yield, the competitive ability and the tannin concentration in 12 cultivars (cv.) of four tanniferous plant species sown in monoculture or in a mixture with a non-tanniferous grass species. Plant performance was observed from sowing in spring 2004 until the last harvest in May 2006. The experiment included *Onobrychis viciifolia* (sainfoin, fam. Fabaceae: ecotype Alvaschein, commercial seed, cv. Visnovsky), *Lotus pedunculatus* (big trefoil, fam. Fabaceae: cv. Grasslands Maku, cv. Barsilvi, cv. Grasslands Sunrise), *Lotus corniculatus* (birdsfoot trefoil, fam. Fabaceae: cv. Odenwälder, cv. Lotar, cv. Oberhaunstädter), *Cichorium intybus* (chicory, fam. Asteraceae: cv. Grasslands Puna, cv. Forage Feast, cv. INIA Lacerta). For the mixtures, we selected *Festuca pratensis* (meadow fescue, fam. Poaceae: cv. Preval), a grass suitable for low input farming with a high nutritive quality but with limited potential as a competitor due to the lack of tiller formation.

The field experiment was done at Agroscope Reckenholz-Tänikon, Research Station ART, in Zurich, Switzerland (47°26' N, 8°30' E). The experimental plants were sown in a split-split plot design with 'plant species' as the main plot factor, 'cultivar' as the subplot factor and 'purely sown vs. mixture' as the sub-subplot factor with 3 blocks (i.e. replicates Gomez & Gomez, 1984). Within each main plot (species), the cultivars were assigned randomly to pairs of sub-subplots with an area of 9 m^2 (1.5 m x 6 m) each. On one of these plots the tanniferous cultivar was sown in monoculture; on its neighbouring plot, it was sown in a mixture with the grass *F. pratensis*. To complete the design, each block contained two grass monocultures of 9 m^2 size (i.e. *Festuca pratensis* cv. Preval). Sowing densities of monocultures of *O. viciifolia*, *L. pedunculatus*, *L. corniculatus*, *C. intybus* and *F. pratensis* corresponded to 180, 18, 18, 4 and 25 kg ha^{-1} of germinable seed, respectively. For the mixtures, we replaced 40 % of the respective tanniferous plant species seed by 40 % of *F. pratensis* seed (i.e. 25 kg ha^{-1} x 40 % = 10 kg ha^{-1}). Per block, one grass sward and all plots containing legumes (i.e. plants of the family Fabaceae with the ability of N_2-fixation by means of symbiotic rhizobia) were fertilized with only 25 kg N ha^{-1} after each harvest. The remaining grass monocultures and the chicory plots were fertilized with 50 kg N ha^{-1} after each harvest.

Swards were harvested twice in the year of sowing (i.e. 2004), four times in 2005 and a

last time in spring 2006. On each of these occasions, three bands of 10 cm width were cut across every sward at 7 cm above ground level for an assessment of the botanical composition of the harvest and a quantification of its tannin concentration[7]. A random sub-sample (one third) of that plant material was immediately put on ice for transportation and dried at 60°C for 48 h as soon as possible, ground to pass through a 0.75 mm sieve, and stored in the dark at room temperature before it was analysed for condensed tannins using a butanol-HCL-assay with a *Lotus pedunculatus* standard (Terrill et al., 1992)[8]. The remaining two thirds of the plant material were separated into 'sown tanniferous species', 'sown *Festuca pratensis*' and 'unsown species'. The separated plant fractions were dried at 100°C for 24 h to calculate their relative contributions to the total dry matter yield. After collecting the botanical samples, swards were harvested by machine[9], 7 cm above ground level and the total dry matter yield of each sward was determined.

4.3.2 Feed palatability

In two consecutive but independent experiments of 20 days duration each, the palatability for wethers of three tanniferous forage plant species (i.e. *Onobrychis viciifolia*, *Lotus corniculatus* and *Cichorium intybus*) was tested in comparison to a control feed consisting of a ryegrass/clover mixture. In a first experiment, feeds were offered as dried forages, in a second experiment they were offered as silages.

O. viciifolia cv. Visnovsky, *L. corniculatus* cv. Oberhaunstädter, C. intybus cv. Grasslands Puna and a ryegrass/white clover/red clover mixture were grown in 2003 at Agroscope Liebefeld-Posieux, Research Station ALP, Posieux (46°46' N, 7° 06' E), Switzerland. The tanniferous forages were topped once at the beginning of July, harvested in August and ensiled in 700 L containers. Second harvests were taken at the end of September (*C. intybus*) or in mid October (*O. viciifolia*, *L. corniculatus*), respectively, and dehydrated at 30°C to a water content below 10 % using a special drying system (Physitech, Wabern, Switzerland) that minimized the loss of tannin-rich plant leaf material. The non-tanniferous control mixture was taken from the fourth cut at the end of September from a ley and was likewise dried or ensiled. The tannin concentrations of the forages were analyzed using a butanol-HCL-assay with a *Lotus pedunculatus* stan-

[7] For a picture of the sampling procedure on the experimental field see page 185 (Appendix B).

[8] The analytical procedure is described in more detail in the section 3.3.3, *Material & Methods*, of the previous chapter on page 56.

[9] For a picture of the harvesting procedure see page 185 (Appendix B).

dard (Terrill et al., 1992)[10].

The experiment involved three groups of adult Oxford wethers (n = 6) corresponding to the three tanniferous forage plant species. On average, wethers were 4.1 $\pm$ 1.8 years old and had a weight of 87.6 $\pm$ 7.2 kg, they were individually housed in pens. Wethers were offered a choice between the respective tanniferous forage and the control feed which were presented simultaneously in two separate boxes. During the first 10 days of each experiment, the diets contained 110 % of the maintenance energy requirement and were given in equal portions twice a day. The maintenance energy requirement (ME_m in MJ; Eqn. 4.1) was calculated as a function of the life weight (LW) of each animal and expressed as metabolizable energy (RAP, 1999):

$$ME_m = 0.38 \times LW^{\frac{3}{4}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.1)$$

During the second 10 days, the sheep received half of the experimental diets (55 % ME_m) at 07:00 h and additionally low-quality hay (100 % ME_m) at 16:00 h. Individual forage specific feed intake was measured twice a day: once after a short evaluation period t (t = 7.5 min for ensiled forage and t = 15 min for dried forage) and once in the afternoon at 16:00 h. The daily palatability index (PI, Eqn. 4.2) was calculated according to the following formula (Ben Salem et al., 1994):

$$PI(t) = I_T(t)/I_C(t) \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.2)$$

where $I_T(t)$ corresponds to the intake of the tanniferous forage eaten during the first t minutes divided by the total intake of tanniferous plants until 16:00 h. Analogously, $I_C(t)$ refers to the intake of the control forage eaten during the first t minutes divided by the total intake of the control forage until 16:00 h. The palatability index assumes that forage selection and intake rates at the beginning of a meal are good criteria to determine palatability of a forage. The palatability index exceeds 100 % when the percentage of the daily consumed tanniferous forage already eaten after t minutes is larger than the corresponding percentage of the control forage. The palatability of the non-tanniferous control feed is defined as 100 %, and tanniferous forages for which the palatability index exceeds 100 % are more palatable than the control feed.

[10] For more details see section *Material & Methods* on page 56.

4.3.3 Antiparasitic activity of *O. viciifolia* hay or silage

The present feeding experiment aimed to assess the efficacy of dried or ensiled forage of *O. viciifolia* against gastrointestinal nematodes in sheep. Twenty-four Swiss White Alpine x Swiss Black-Brown Mountain lambs (10 females and 14 males) were artificially infected with a single dose of *Cooperia curticei* and *Haemonchus contortus* larvae. The lambs were between 2.5 and 3 months old and had a mean liveweight of 33 kg at the start of the experiment. Twenty-eight days post infection, the lambs were allocated to 4 comparable groups according to sex, bodyweight and faecal egg counts. For 16 days the lambs were fed ad libitum with either *O. viciifolia* or a corresponding isoproteic and isoenergetic non-tanniferous control forage each as regular hay or silage, respectively. Faecal egg counts per gram dry faeces were performed twice a week. Tannin concentrations of the forages were analyzed using a butanol-HCL-assay with a *Lotus pedunculatus* standard (Terrill et al., 1992)[11].

4.4 Results & Discussion

4.4.1 Agronomic performance of tanniferous forage plants

Onobrychis viciifolia, *Lotus corniculatus* and *Cichorium intybus* germinated and established well in 2004. In contrast, all cultivars of *Lotus pedunculatus* were soon outcompeted after an initially promising germination, mainly by unsown *Trifolium repens*. As a result, the dry matter proportions of *L. pedunculatus* was below 20 % DM of the yield for any of the cultivars in 2005 (Table 4.1 on page 87), even when sown as a monoculture. Therefore, the following text will concentrate on results of *O. viciifolia*, *L. corniculatus* and *Cichorium intybus* only[12].

The cultivars of *O. viciifolia*, *L. corniculatus* and *C. intybus* yielded between 9.9 and 13.0 t DM ha^{-1} when sown in a monoculture (Table 4.1 on page 87). Sowing the tanniferous plants in a mixture with *Festuca pratensis* increased the total yield markedly in the case of *O. viciifolia* (yields of mixtures ranged between 16.4 and 16.5 t DM ha^{-1}), and even more so in *L. corniculatus* (yields of mixtures ranged between 17.3 and 18.4 t DM ha^{-1}) but only slightly in *C. intybus* (yields of mixtures ranged between 12.5 and 12.8 t DM

[11] For a more detailed description of the analytical procedure see the section 3.3.3, *Material & Methods*, on page 56.

[12] For pictures of the swards see page 184 (Appendix B).

ha^{-1}). In the case of *O. viciifolia* and *L. corniculatus* yields of mixtures clearly exceeded the yields of the respective monocultures of the tanniferous plant species as well as that of the grass monoculture. This transgressive overyielding resulted most likely from N_2-fixation (Jacot et al., 2000) of the tanniferous legume species and from a spatial and temporal niche complementation (Loreau, 2000; Kirvan et al., 2007) with regard to growth.

For *O. viciifolia*, *L. corniculatus* and *C. intybus*, dry matter proportions of sown species in the yield ranged between 38 and 100 % when the tanniferous cultivars were sown in a monoculture but between 89 and 100 % when they were sown in a mixture with *F. pratensis* (Table 4.1). In other words, *F. pratensis* helped to reduce the invasion of weed and other unsown species both in terms of absolute and relative amounts of weed. However, the grass also reduced the proportion of sown tanniferous plant material in the harvest from between 38 to 100 % in monoculture to 6 to 94 % in mixtures and, as a consequence, tannin concentrations in the harvest of mixtures were diluted accordingly (Table 4.1). Between species, differences in tannin concentrations at the yield level (Table 4.1) were much smaller than might have been expected from tannin concentrations of the tanniferous species alone (Fig. 4.1 on page 88). While the yield of species with high tannin concentrations (i.e. *O. viciifolia* and *L. pedunculatus*) was heavily diluted by non-tanniferous plant material, the dilution was less severe in *L. corniculatus* which has intermediate tannin concentrations, and almost non-existent in the case of *C. intybus* which has very low tannin concentrations.

Cultivar Visnovsky was clearly the most promising among the tested *O. viciifolia* cultivars. Visnovsky had the highest yield (when sown as a monoculture: 13.0; when sown as a mixture: 16.5 t ha^{-1} DM) and the highest dry matter proportions of the sown plant species (monoculture: 76, mixture: 98 % DM), the highest dry matter proportion of tanniferous plant material in the yield (monoculture: 76, mixture: 36 % DM) and therefore also the highest tannin concentrations (monoculture: 24, mixture: 12 g kg^{-1} DM). For *L. corniculatus*, cultivar Lotar performed slightly better than cv. Oberhaunstädter and cv. Odenwälder. Lotar achieved a yield of 11.0 t ha^{-1} in monoculture and 18.4 t ha^{-1} in a mixture with *Festuca pratensis*. The proportion of tanniferous plants was 77 % in monoculture and 32 % when sown in a mixture and the tannin concentration of the yield (monoculture: 18, mixture: 10 g kg^{-1} DM) was only slightly lower than in *O. viciifolia*. Among the tested cultivars of *C. intybus*, the cultivar Puna clearly performed best. The other cultivars produced a large amount of stems, especially in the second year, adding to the dry matter yield and the dry matter yield proportions but supposedly reducing the

Table 4.1: *Total yield, dry matter proportion of sown species, dry matter proportion of tanniferous species and tannin concentration of total yield harvested in the year 2005 (mean se; n = 3, swards were cut four times in 2005). All cultivars were sown in 2004 either as pure stands or in a mixture with* Festuca pratensis.

	Total yield (t ha^{-1} y^{-1} DM)		**Sown species** (% of total yield)		**Tanniferous species** (% of total yield)		**Tannin conc. of yield** (g CT kg^{-1} DM)	
	pure	**mixture**	**pure**	**mixture**	**pure**	**mixture**	**pure**	**mixture**
O. viciifolia†								
Alvaschein	11.0± 0.3	16.4± 1.1	46 ± 8	90 ± 2	46 ± 8	6 ± 1	21 ± 4	4 ± 1
Commercial seed	11.1± 0.2	16.4± 1.1	38 ± 13	96 ± 1	38 ± 13	6 ± 3	17 ± 3	4 ± 2
Visnovsky	13.0± 1.2	16.5± 1.6	76 ± 6	98 ± 1	76 ± 6	36 ± 3	24 ± 2	12 ± 1
L. pedunculatus†								
Maku	7.7±0.3	18.1±1.0	19±3	88±1	19±3	5±2	13±3	4±1
Barsilvi	9.0±1.1	17.1±0.1	2±1	76±1	2±1	1±1	4±1	1±1
Sunrise	8.6±1.1	17.6±1.0	5±1	83±3	5±2	1±1	5±1	2±1
L. corniculatus†								
Odenwälder	9.9±1.0	18.0±1.2	70±12	95±3	70±12	21±9	17±1	6±1
Lotar	11.0±1.0	18.4±1.1	77±5	97±1	77±5	32±9	18±2	10±2
Oberhaunstädter	10.0±1.5	17.3±1.1	77±6	89±4	77±6	39±10	15±1	8±1
C.intybus‡								
Puna	12.8±1.0	12.8±0.6	100±0	100±1	100±1	94±2	5±1	4±1
Forage Feast	9.9±0.5	12.6±0.7	99±1	98±2	99±1	72±9	5±1	3±1
Lacerta	12.1±1.2	12.5±1.5	100±0	100±1	100±1	87±1	2±1	3±1
F. pratensis								
Preval‡	13.8±0.8	—	92±2	—	—	—	—	—
Preval†	14.2±1.2	—	82±1	—	—	—	—	—

† 100 kg N y^{-1} ‡ 200 kg N y^{-1}

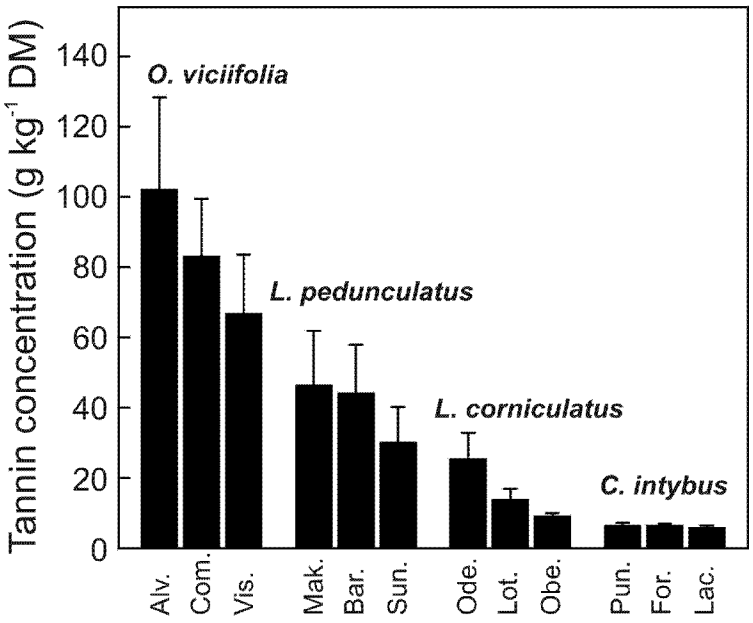

Figure 4.1: *Tannin concentrations of harvestable aboveground biomass (> 7 cm; mean se; n = 3) of twelve field grown tanniferous cultivars:* O. viciifolia*: ecotype Alvaschein, commercial seed, cv. Visnovsky.* L. pedunculatus*: cv. Grasslands Maku, cv. Barsilvi, cv. Grasslands Sunrise.* L. corniculatus*: cv. Odenwälder, cv. Lotar, cv. Oberhaunstädter.* C. intybus*: cv. Grasslands Puna, cv. Forage Feast, cv. INIA Lacerta. All cultivars were collected on May 8, 2006.*

forage quality.

Figure 4.2 on the facing page displays the dry matter proportion of tanniferous plant material in each harvest and the corresponding tannin concentration for the most promising species and cultivars in this study (i.e. *O. viciifolia* cv. Visnovsky and *L. corniculatus* cv. Lotar). In both purely sown and mixed stands of *O. viciifolia* or *L. corniculatus*, respectively, fluctuations of dry matter proportions between tanniferous plants and non-tanniferous plant species were considerable. For example, in monocultures of *O. viciifolia* cv. Visnovsky, the proportion of tanniferous plants was 80 % in August 2004, decreased to 55 % DM in October of the same year but recovered to almost 100 % DM in July of the following year. Shifts in the relative contribution of tanniferous plant material to total yield were reflected in the seasonal dynamics of tannin concentrations in the harvest. Therefore, we conclude that shifts in dry matter proportions and the competitive abilities of the tanniferous plant species are major drivers of seasonal fluctuations of tannin concentrations in the harvest.

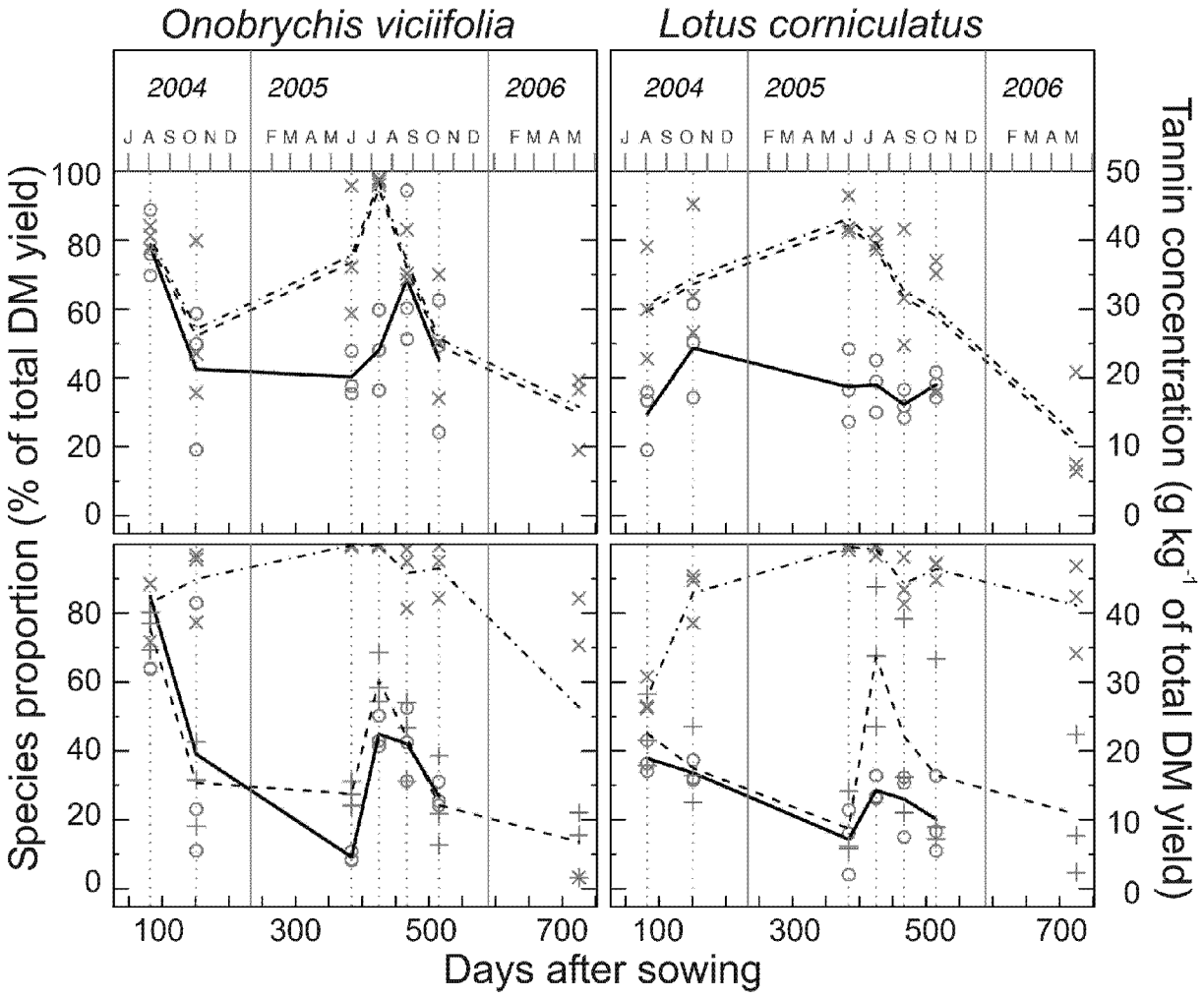

Figure 4.2: *Mean proportion of sown species (dash-dotted line, n = 3), mean proportion of tanniferous species (dashed line, n = 3) as percentages of total dry matter yield, respectively, and mean tannin concentrations of individual yields (solid line, n = 3) in the course of the experiment. Upper row: Purely sown stands of* Onobrychis viciifolia *cv. Visnovsky,* Lotus corniculatus *cv. Lotar. Lower row: The same species and cultivars as above but sown in a mixture with the grass* Festuca pratensis*. Original data on the plot level are presented as symbols (i.e. crosses = sown species, pluses = tanniferous species, open circles = tannin concentration). Compare to the pictures on page 185 (Appendix B).*

4.4.2 Feed palatability

O. viciifolia had the highest concentration of condensed tannins followed by *L. corniculatus*, while the tannin concentration of *C. intybus* was low. For all the investigated

plant species, tannin concentrations (g kg^{-1} DM) were higher in silage (*O. viciifolia*: 100; *L. corniculatus*: 41; *C. intybus*: 14) than in dried plant material (*O. viciifolia*: 92; *L. corniculatus*: 26; *C. intybus*: 11).

Offered as dried forages (Fig. 4.3 on the next page), palatability indices during the first 10 days were higher for *O. viciifolia* (PI: 91.2 $\pm$ 23.9 %) and *C. intybus* (PI: 84.3 $\pm$ 23.0 %) than for *L. corniculatus* (PI: 65.5 $\pm$ 21.8 %). However, mainly due to very low initial values, none of the tanniferous forages was as palatable as the control forage when averaged over the first ten days (the PI of the control is defined as 100 %). By increasing the energy and nutrient supply ten days after the start of the experiment, we expected the wethers to indicate the palatabilities of the different feeds more differentiatedly. However, during the second ten days the palatability indices of all the tanniferous forages approximated that of the control (i.e. 100 %) to an even greater extent (average PI: *O. viciifolia*: 95.6 $\pm$ 2.9 %; *C. intybus*: 102.9 $\pm$ 13.5 %; *L. corniculatus*: 100.2 $\pm$ 13.1 %). Hence, the palatability of the dried tanniferous forages was very similar to that of the dried ryegrass/clover mixture.

Fed as ensiled forages (Fig. 4.3 on the facing page), the palatabilities of *O. viciifolia* (151.9 $\pm$ 81.9 %) and *C. intybus* (121.2 $\pm$ 69.0 %) were clearly higher than that of the control feed during the first ten days of the experiment. Only *L. corniculatus* (PI: 77.7 $\pm$ 33.3 %) had a palatability index lower than the ensiled control forage. During the second ten days, *O. viciifolia* (on average: 159.6 $\pm$ 51.2 %) remained more palatable than the control forage. Palatability of *C. intybus* (100.6 $\pm$ 16.2 %) and *L. corniculatus* (101.0 $\pm$ 44.5 %), however, were similar to that of the control forage.

The average palatability indices showed a high variation both among individual animals and between days. This variation was greater for ensiled than for dried forages, possibly due to the shorter evaluation time for the ensiled forage (7.5 min for ensiled forages vs.15 min for dried forages). In the case of dried tanniferous forages, palatability was initially low compared to controls. Already two to three days after the start of the experiment, however, the palatability of dried tanniferous forages did not differ from that of the familiar non-tanniferous control forage. Within this short period of time, adaptations in the wether's physiology or rumen flora, to avoid potentially detrimental effects of condensed tannins, are highly unlikely. It seems more plausible that the initially low but strongly increasing palatability indices indicate beneficial postingestive feedbacks or simply reflect a sensory customization to the unfamiliar diets. Wethers needed no time

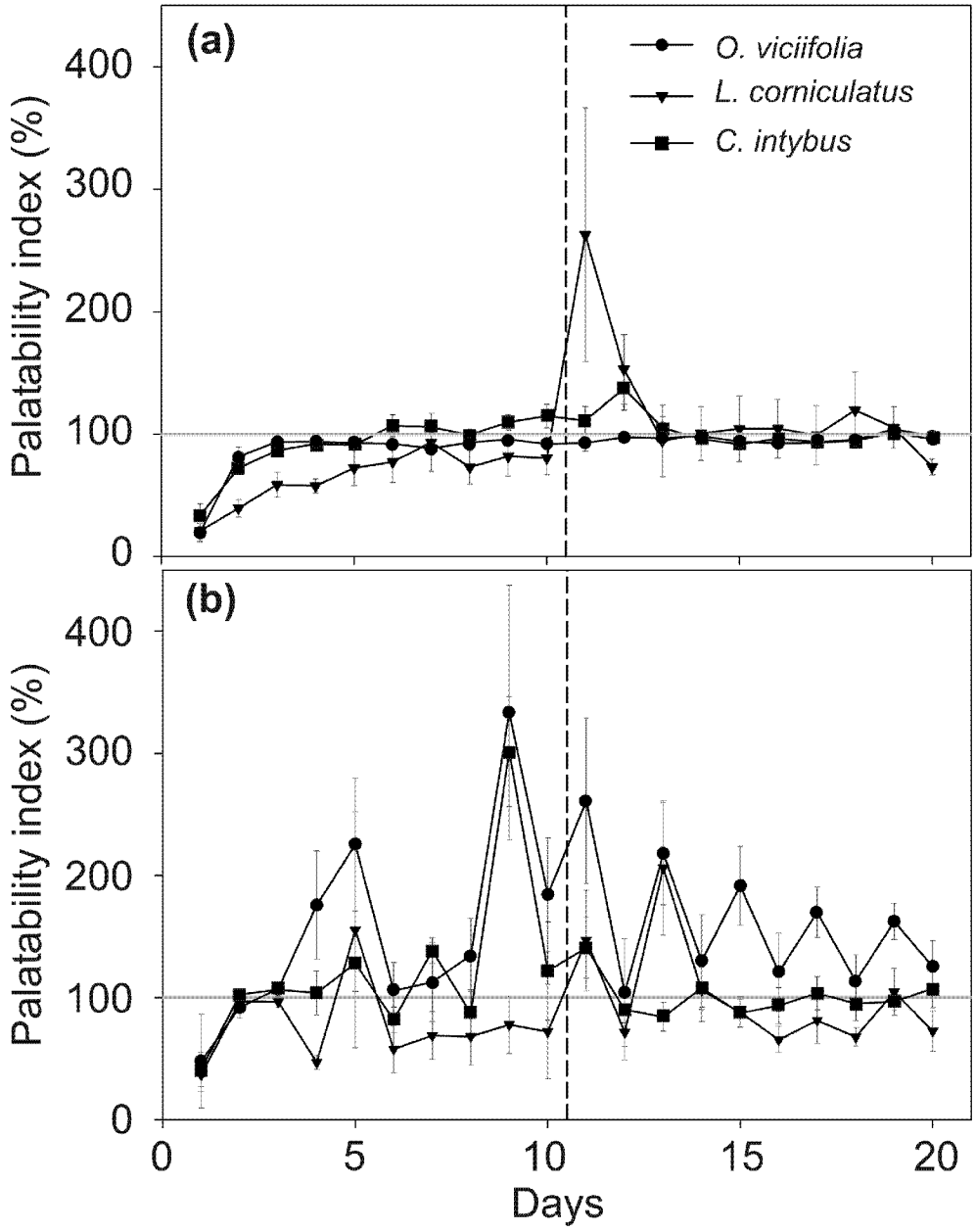

Figure 4.3: *Palatability indices (PIs) of (a) dried or (b) ensiled tanniferous forage plants compared to a dried or ensiled non-tanniferous ryegrass/clover mixture (control), respectively. The palatability index of the control is defined as 100 %. Tanniferous forages for which the palatability index exceeds 100 % are more palatable to wethers than the control. From day one to ten, feeds covered 110 % of the maintenance energy requirement. After day ten (dashed line), energy supply was increased to 155 % of the maintenance energy requirement (see section Material & Methods).*

to become accustomed to ensiled tanniferous forages. They were at least as palatable as the control feed immediately after the start of the experiment. It is concluded that after two to three days, and independent of the conservation method used, all tested tanniferous forage plants were at least similarly palatable as an equally conserved ryegrass/clover mixture. Ensiled *O. viciifolia* had the highest tannin concentrations of all the tested diets and regardless of its elevated tannin concentrations was clearly more palatable to the wethers than an ensiled ryegrass/clover mixture. Therefore, *O. viciifolia*, especially in its ensiled form, is considered the most promising candidate for a practical application against gastrointestinal nematodes.

4.4.3 Antiparasitic activity of *O. viciifolia* hay or silage

The tannin concentrations of *O. viciifolia* hay and silage were 62 g kg^{-1} DM and 41 g kg^{-1} DM, respectively. Compared to their respective control groups, marked reductions in the combined faecal egg counts of *Haemonchus contortus* and *Cooperia curticei* were observed when *O. viciifolia* was fed as hay or silage (Fig. 4.4 on the next page). Within the 16-day feeding period, faecal egg counts decreased by 58 % when lambs were fed with *O. viciifolia* hay whereas they increased by 43 % when lambs were fed with the control hay. For *O. viciifolia* silage, faecal egg counts were reduced by 37 % whereas in the corresponding control group faecal egg counts increased by 16 %.

In comparison to the results of freshly administered *O. viciifolia* (Heckendorn et al., 2007), the anti-parasitic properties of *O. viciifolia* were largely preserved in both silage and hay leading to a substantial decrease in worm egg excretion in faeces. Our results imply that the ensuing pasture contamination with infective larvae will decrease considerably and reinfections will be reduced when sheep are fed with *O. viciifolia*.

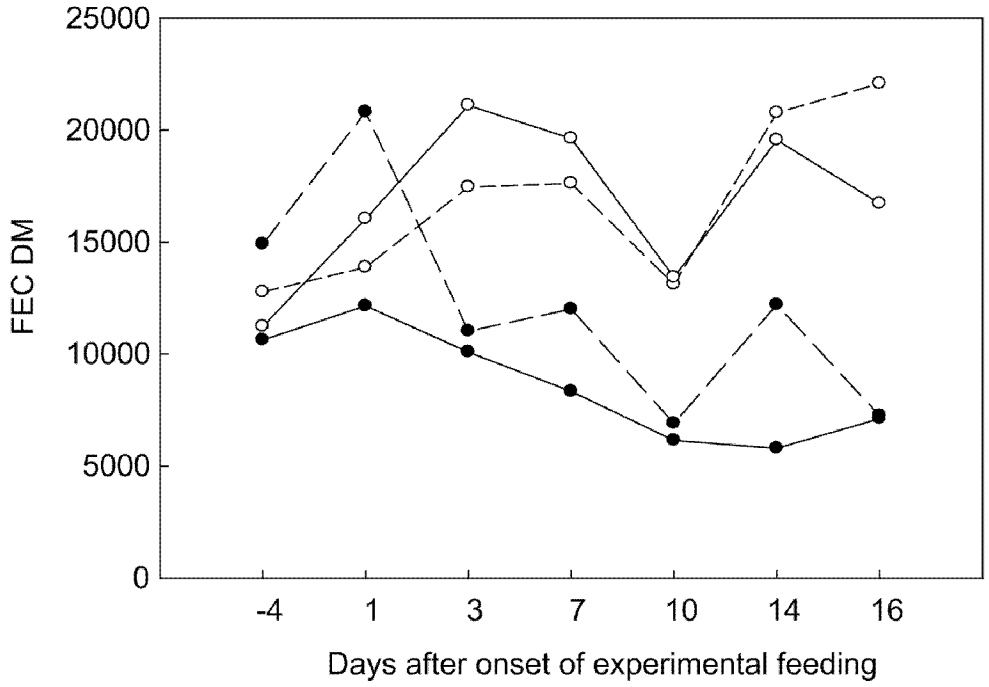

Figure 4.4: *Mean parasite egg counts per gram dried faeces (FEC DM; n = 6) of lambs fed either with* O. viciifolia *(closed symbols) or non-tanniferous control forage (open symbols), each administered as hay (dashed line) or silage (solid line), respectively.*

4.5 Synthesis

O. viciifolia (cv. Visnovsky), *L. corniculatus* (cv. Lotar) and C.intybus (cv. Grasslands Puna) appeared particularly suitable for cultivation under the given temperate climatic conditions, whereas *L. pedunculatus* was competitively weak, possibly because this species prefers more humid than 'average' agronomic conditions. Similar performances of these species have been observed in an independent cultivation trial at Frick, Switzerland (Heckendorn et al., 2007). With regard to their tannin concentrations, only *O. viciifolia* and *L. corniculatus* seemed promising candidates for the control of gastrointestinal nematodes in ruminants whereas tannin concentrations of *C. intybus* were very low. Nevertheless, *C. intybus* has repeatedly been reported to have anthelmintic and other desirable properties possibly related to elevated concentrations of other secondary metabolites (e.g. sesquiterpene lactones) found in this species (Hoste et al., 2006).

Our field experiment demonstrated that the competitive abilities of even the most promising tanniferous candidate plants (i.e. *O. viciifolia* cv. Visnovsky and *L. corniculatus* cv. Lotar) were suboptimal. As a consequence, seasonal fluctuations of tannin con-

centrations can, to a large extent, be attributed to shifts in the relative contribution of tanniferous plant material to total yield, even in purely sown swards. Pronounced short-term fluctuations in tannin concentrations of harvestable biomass (e.g. *O. viciifolia*, + 79 % within 17 days; (Heckendorn et al., 2007)) can result from differences in momentary growth rates between sown tanniferous plant species and their (sown or unsown) non-tanniferous neighbours. Additionally, fluctuations of tannin concentrations in pure stands can result from shifts in leaf/stem ratios of tanniferous forage plants during their development (Häring et al., 2007). This implies that with regard to the suggested breeding (Aerts et al., 1999) or even genetic engineering (Marles et al., 2003) of tannin-rich plants, a high competitive ability and a large leaf to stem ratio (Häring et al., 2007) are parameters likely to be just as important in enhancing the tannin concentration of harvestable biomass as an elevated intrinsic tannin concentration of the leaves of a cultivar.

With regard to the cultivation and management of tanniferous forage plants, mixtures were found to have clear advantages in relation to the total dry matter yield and in suppression of unsown species. Future research should focus on how to profit from the advantages of mixtures without suppressing tanniferous plants and lowering tannin concentrations. Possible options are (i) to lower nitrogen input to increase the competitive advantage of the tanniferous legume relative to the grass competitor, (ii) to reduce sowing densities of the grass, or (iii) to test mixtures with other grasses (e.g. short-bladed and slow-establishing grasses such as *Agrostis alba* or *Festuca rubra*). Alternatively, one could try to enhance performance and reduce weed invasion in monocultures of tanniferous forages, for example by (iv) increasing sowing densities or (v) by adapting cutting frequencies to the tanniferous species.

Parallel to optimizing the cultivation and concentration of condensed tannins in the target candidates, investigations of the acceptance of these plants by sheep are of major importance. The feeding and palatability experiment showed that two to three days after the start of the experiment, the palatability of tanniferous forages was at least similar to a ryegrass/clover control, independent of the conservation method used. Regardless of its comparatively high tannin concentration, ensiled *O. viciifolia* was the most palatable among the tested forages. There was no evidence of negative side effects on the wethers from any of the tanniferous forages. Across species, the palatability of the different forages appeared unrelated to their tannin concentrations. If condensed tannins as 'plant defensive compounds' (Bryant et al., 1983; Herms & Mattson, 1992; Stamp, 2003) should deter herbivores, they do not seem to have acted through a depressing effect on palatability or short term physiological feedbacks in our experiment. It is also

possible that the apparent lack of a 'plant defensive effect' of condensed tannin in this study was caused by interactions with other nutrients (Waterman & Mole, 1994; Villalba et al., 2002) or different chemical structures and 'potency' of tannins in the tested plant species (5). In this context, the multitude of potentially influential chemical features of tannins and the great variety of suggested methods to analyze them contrasts with the fact that we still lack a simple but useful principle to interlink the chemical structure and the biological activity of tannins (Waterman & Mole, 1994; Kraus et al., 2003; Mueller-Harvey, 2006).

Apart from the general interest in the potential advantages and disadvantages of tanniferous forages for ruminant metabolism, the present study was primarily aimed at preparing a pathway towards their use against gastrointestinal nematodes. Administering hay or silage of *O. viciifolia* to lambs co-infected with *Haemonchus contortus* and *Cooperia curticei* reduced the combined faecal egg output markedly compared to the controls. As trichostrongylidosis (i.e. the disease caused by gastrointestinal nematodes) is a pasture borne disease, a reduced contamination of the pasture with parasite eggs and infectious L3 larvae is likely to diminish the risk of renewed infections. Besides the effect on faecal egg output, there is strong evidence for a direct lethal effect of *O. viciifolia* on *Haemonchus contortus* (Heckendorn et al., 2006, 2007), the species with the highest pathogenicity among the gastrointestinal nematodes of temperate and (sub)tropical regions.

4.6 Conclusions

The satisfactory yield and competitive ability of *O. viciifolia*, its high palatability despite its elevated tannin concentration, and its efficacy against gastrointestinal parasites even in a conserved form would appear to make this tanniferous plant species an ideal candidate for the implementation of a non-pharmaceutical control strategy against gastrointestinal parasites. With regard to forage conservation, ensiling seems preferable to drying because it minimizes the loss of tannin-rich leaves, allows forage conservation relatively independent of the weather and, according to our results, does not diminish the palatability to sheep or the efficacy against intestinal parasites. Further research is needed to improve our understanding of the relationship between the chemical features of the condensed tannins and their biological activity. In particular, it is essential to clarify the mode of action by which condensed tannins exert antiparasitic effects in sheep. Future studies should aim to enhance efficacy against gastrointestinal nematodes by

focussing on the possibilities of increasing concentrations of condensed tannins in the offered forage without reducing the palatability or nutritional quality of the feed. Finally, a control strategy against gastrointestinal nematodes based on condensed tannins needs to be adapted to optimize its technical and economical practicability for employment and acceptance on private sheep farms.

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Veterinary Parasitology 146 (2007) 123–134
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Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemonchus contortus* and *Cooperia curticei*

Felix Heckendorn [a,b,*], Dieter Adrian Häring [c], Veronika Maurer [a], Markus Senn [b], Hubertus Hertzberg [a,d]

[a] *Department of Veterinary Parasitology, Research Institute of Organic Agriculture, Ackerstrasse, CH-5070 Frick, Switzerland*
[b] *Physiology and Animal Husbandry, Institute of Animal Sciences, ETH Zurich, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland*
[c] *Research Station Agroscope Reckenholz—Tanikon ART, Reckenholzstrasse 191, CH-8046 Zurich, Switzerland*
[d] *Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland*

Received 24 November 2006; received in revised form 15 January 2007; accepted 16 January 2007

Chapter 5

Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemonchus contortus* and *Cooperia curticei*

HECKENDORN F.[1] HÄRING D.A.[23], MAURER M.[1]SENN M.[4], HUBERTUS HERTZBERG[15]

Published in *Veterinary Parasitology* 146 (1–2): pp. 123–134, 2007.

This chapter is the result of a cooperation between the module *Plant Sciences* and the module *Parasitology* within the *Tannin-Project*. It investigates the antiparasitic effect of feeding fresh tanniferous forage and aimed at establishing the first in vivo dose-response relationship of the antiparasitic effect of condensed tannins against gastrointestial nematodes in sheep. The author of this thesis contributed to the current chapter by analysing the chemical and botanical compostition of the various forages, by providing help with the statistical analysis and the graphical presentation of the data as well as by an active participation in the thinking and writing process.

[1]Research Institute of Organic Agriculture, Frick
[2]Agroscope Reckenholz-Tänikon Research Station ART, Zurich
[3]Institute of Plant Sciences, ETH Zurich
[4]Institute of Animal Sciences, ETH Zurich
[5]Institute of Parasitology, University of Zurich

5.1 Abstract

We investigated direct anthelmintic effects associated with the feeding of fresh tanniferous forages against established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs. Twenty-four parasite naive lambs were inoculated with a single dose of infective larvae of these two parasites 27 days prior to the start of the feeding experiment. Lambs were individually fed with either chicory (*Cichorium intybus*), birdsfoot trefoil (*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia*) or a ryegrass : lucerne mixture (control) for 17 days. Animals where then united to one flock and subjected to control feeding for another 11 days to test the sustainability of potentially lowered egg excretion generated by tanniferous forage feeding.

When compared to the control, administration of all tanniferous forages was associated with significant reductions of total daily faecal egg output specific to *H. contortus* (chicory: 89 %; birdsfoot trefoil: 63 %; sainfoin: 63 %; all tests $P < 0.05$) and a tendency of reduced *H. contortus* worm burden (chicory: 15 %; birdsfoot trefoil: 49 % and sainfoin: 35 % reduction). Irrespective of the condensed tannin (CT) containing fodder, no anthelmintic effects were found against *C. curticei*. Cessation of CT-feeding followed by non-CT control feeding did not result in a re-emergence of faecal egg counts based on faecal dry matter (FECDM) in any group, suggesting that egg output reductions are sustainable. The moderate to high concentrations of CTs in birdsfoot trefoil (15.2 g CTs kg^{-1} dry matter (DM)) and sainfoin (26.1 g CTs kg^{-1} DM) were compatible with the hypothesis that the antiparasitic effect of these forages is caused by their content of CTs. For chicory (3 g CTs kg^{-1} DM), however, other secondary metabolites need to be considered. Overall, birdsfoot trefoil and in particular sainfoin seem promising candidates in contributing to an integrated control strategy against *H. contortus* not only by mitigating parasite related health disturbances of the host but also by a sustained reduction of pasture contamination.

Keywords

chicory, birdsfoot trefoil, sainfoin, *Haemonchus contortus*, *Cooperia curticei*, condensed tannins, sheep

5.2 Introduction

The wide spread development of anthelmintic resistant populations of gastro-intestinal nematodes (GIN) during the last decade (Jackson & Coop, 2000) and the requirements for reduced anthelmintic input in organic production systems (Waller & Thamsborg, 2004) necessitates the development of alternative, non chemical control strategies against GIN. Recent research has suggested that forages containing condensed tannins (CTs) may offer a promising alternative approach for the control of GIN. In several experiments, the consumption of tanniferous forages was associated with reduced levels of GIN parasites and improved performance of small ruminants (Min & Hart, 2002). It has been hypothesised that the effect of CTs against GIN might be indirect, by long term improvement of host immunity due to an increased protein availability or direct, by short term affection of several biological key processes of parasites (Hoste et al., 2006). In vitro and in vivo experiments with sheep and goats in which the short-term experimental design did not permit the development and expression of host immune responses support the hypothesis of a direct effect of CTs against GIN (Molan et al., 2000b, 2003; Paolini et al., 2003a, 2004; Heckendorn et al., 2006). These studies, however, also pointed out that the anthelmintic effect of tanniferous forages is variable, depending on the GIN parasite species, the parasitic stage, the CT-containing forage plant species and probably also on the host species used. For example, lambs carrying adult *Teladorsagia circumcincta* worms and fed on fresh CT-forages, such as chicory (*Cichorium intybus*) and sainfoin (*Onobrychis viciifolia*) had lower levels of this parasite compared to those receiving non CT-containing control feeds (Marley et al., 2003; Thamsborg et al., 2004; Tzamaloukas et al., 2005). With the same forages, however, essentially no effect was observed against the intestinal GIN species *Trichostrongylus colubriformis* (Athanasiadou et al., 2005). It is unclear, whether these findings are evidence of true species specificity in the sense that CTs inhibit or suppress biological processes in *T. circumcincta* but not in *T. colubriformis* or whether they are a result of the different location of these parasites in the gastrointestinal tract. Overall, the accumulated data suggest that one important direction in this field of research must be the investigation of individual GIN species responses towards individual CT-containing forages in order to learn more about the specific direct action of CTs (Hoste et al., 2006).

In this experiment, we intended to compare the efficacy of four tanniferous forages with respect to faecal egg excretion and worm burden of two GIN species in lambs under identical experimental conditions in order to get further insights in parasite and forage specific effects. As a first experimental parasite, we chose *Haemonchus contortus*, an

abomasal sheep parasite of global importance with which only few studies have been conducted so far (Paolini et al., 2003b; Min et al., 2004; Heckendorn et al., 2006; Lange et al., 2006). To the best of our knowledge, the administration of fresh CT-containing forages to small ruminants infected with *H. contortus* has never been evaluated yet. As a second parasite *Cooperia curticei* was included in the study, an intestinal sheep GIN of regional importance in Europe (Rehbein et al., 1996, 1998).

As tannin containing forages we chose chicory (*Cichorium intybus*), birdsfoot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*) and sainfoin (*Onobrychis viciifolia*). It is known from former studies that these forages differ widely in their tannin content. By quantifying the CT-content of each forage and by recording the individual feed intake of every lamb, we aimed to establish a dosage-effect relationship of CTs against *H. contortus* and *C. curticei* with CTs from field grown plants. A further objective of the study was to investigate whether the antiparasitic effect of CTs is a direct result of the elimination of established GIN or if the effect is limited to a temporary reduction in parasite fecundity. A number of studies found that for *T. colubriformis* and *H. contortus*, the observed reductions in egg excretion disappeared when CT-administration was stopped (Athanasiadou et al., 2000; Min et al., 2005; Lange et al., 2006), suggesting that CTs temporally reduced the female worm fecundity. Other experiments including a variety of CT-containing plants and GIN, however, concluded that reductions in faecal egg count (FEC) were mainly associated with reductions of adult worms (Niezen et al., 1995; Thamsborg et al., 2004; Heckendorn et al., 2006).

5.2.1 Animals, Materials and Methods

2.1. Forage cultivation In early spring 2004, four 0.25 ha plots were sown as pure stands of chicory (*Cichorium intybus*, cv. Grasslands Puna), birdsfoot trefoil (*Lotus corniculatus*, cv. Odenwälder), sainfoin (*Onobrychis viciifolia*, cv. Visnovsky), big trefoil (*Lotus pedunculatus*, cv. Barsilvi) or with a ryegrass : lucerne mixture at FiBL, Frick, Switzerland. Sowing rates were adjusted for germination percentages of the seed samples and corresponded to 32 kg ha^{-1} of germinable seed for ryegrass : lucerne, 18 kg ha^{-1} for birdsfoot trefoil, 180 kg ha^{-1} for sainfoin, 11 kg ha^{-1} for big trefoil and 4 kg ha^{-1} for chicory. All plots except for big trefoil were cut in mid-May and the re-growths were used as experimental feeds in late June. Big trefoil at this stage had to be excluded from the study, because this species was outcompeted almost completely, resulting in swards that contained less than 1 % DM of this forage. At the start of the experiment,

sainfoin and birdsfoot trefoil were at the 50 % flowering stage whilst chicory was still in a vegetative stage.

5.2.2 Animals

Twenty-four Swiss White Alpine $\times$ Swiss Black-Brown Mountain lambs were used in the study. They were penned indoors under conditions that minimized nematode infection. Lambs were given a multivalent vaccination against clostridia infections at approximately 5 and 10 weeks of age and were treated with levamisole (Endex 8.75 %, 7.5 mg kg^{-1} body weight) to ensure trichostrongle-free conditions. After weaning, at an age of 3.5 - 4 months, the animals were accustomed to fresh fodder during a 4 week period prior to the start of the experiment. At the start of the feeding trial the lambs had a mean live weight of 22.4 $\pm$ 0.6 kg.

5.2.3 Parasite isolates and experimental infection

Infective larvae of *H. contortus* and *C. curticei* were cultured from faeces of monospecifically infected donor lambs according to standard procedures. Parasite isolates were kindly provided by Merial, Germany. Twenty-seven days prior to the start of the feeding experiment all lambs were inoculated with a single dose of 7000 third stage larvae of *H. contortus* and 15000 third stage larvae of *C. curticei*. The severity of the infection in all lambs was quantified 24 days post infection (p.i.) i.e. 3 days prior to the start of the CT-feeding experiment, respectively, by means of FEC.

5.2.4 Experimental design

The CT-feeding experiment was conducted in a randomised complete block design. On day 24 p.i., the lambs were allocated to six blocks according to their initial FEC (see above). Within each block, the four sheep were randomly assigned to the four experimental feeds: chicory, birdsfoot trefoil, sainfoin or the non-tanniferous control feed (i.e. a ryegrass : lucerne mixture). From day 27 p.i. animals were fed with their respective experimental feed for 17 consecutive days. After the CT-feeding period, lambs were united to one flock and subjected to group feeding with non-tanniferous control feed for another 11 days in order to test whether potential effects on FEC of the different feeds on lambs are reversible. Finally lambs were slaughtered to determine the adult worm burden in their intestines.

5.2.5 Forage administration, feed intake and live weight

The daily required portions of each of the 4 forages were harvested early every morning and stored at 4° C for later use. Sub-samples of each forage were taken immediately after harvest and analysed daily for dry weight. Fresh fodder was offered to the lambs ad libitum 3 times a day (morning, afternoon and evening) and individually weighed before administration. Equally, feed refusals and spillage were measured 3 times a day. Thus, daily fodder intake was known for each individual sheep. In addition, live weights of animals were recorded every week.

5.2.6 Feed analysis

The botanical composition and CT-contents of the experimental feeds were analysed on day 3, 8 and 13 of the 17 day CT-feeding period. In order to describe the botanical composition of each feed, harvest samples were separated according to plant species, dried and the relative contribution of different functional plant groups to total yield were calculated. Corresponding samples were freeze dried and analysed for condensed tannins by the method described in Terrill et al. (1992). Nutritive values, such as net energy, protein content and in vitro-digestibility were determined from a bulk sample of daily collected and lyophilized sub-samples at the end of the experiment using standard procedures. Organic matter digestibility (OMD) of the experimental feeds was determined in vitro according to Tilley & Terry (1963).

5.2.7 Parasitological procedures and measures

During the individual CT-feeding period and the subsequent group feeding period, individual faecal samples were taken from the rectum every 2-4 days. Faecal samples were processed immediately for FEC (Schmidt, 1971) and dry matter (DM) content. The DM of the faeces was calculated from a 3 g sub-sample dried in a force-draught oven at 105°C for 16 h. Since different feeds can influence faecal dry matter, faecal egg counts were expressed as the number of eggs per gram of dried faeces (FECDM) as described in Heckendorn et al. (2006). During the CT-feeding period, pooled quantitative faecal cultures were prepared group wise using a 2 g sub-sample of fresh faeces from every lamb. Prior to cultivation, the faecal material was homogenized thoroughly in order to uniformly distribute the eggs and pre-culture FEC were performed. Per culture, 12 g of faecal material was placed in a polystyrene container and incubated for 10 days at 20°C

under conditions that maximised humidity. Post-culture, larvae were extracted for 24 h using a Baermann apparatus and transferred to Falcon tubes. After a 6 hour storage period at 4° C, excess liquid was removed by siphoning and the concentrated larvae were transferred to tissue culture flasks and stored at 6° C until processing. Total numbers of infective larvae were counted in 500 μl aliquots (10 times 50 μl) and the mean counts extrapolated to the total culture volume. FECDM specific for *H. contortus* and *C. curticei* were calculated as described in Heckendorn et al. (2006). Blood samples were taken weekly during the experiment in order to monitor packed cell volume (PCV) as a parameter reflecting the severity of the parasite infection. A PCV level below 15 was defined as exclusion criterion.

As during CT-feeding the dry matter of daily feed intake (DMDI) and the percentage of in vitro digestibile organic matter (OMD) were known for each feed, the dry matter of total daily faecal output (TDFO; Eqn. 5.1; Mayes et al. 1986) could be estimated for each individual sheep:

$$TDFO \quad (\text{g DM}) \quad = \quad DMDI \quad \times \quad (1 - OMD) \quad . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; (5.1)$$

Subsequently, an estimate of the total daily faecal egg output ($TDFEO$: Eqn. 5.1) was obtained by multiplying the total daily faecal output (TDFO) by the faecal egg counts per gram dried faeces (FECDM):

$$TDFEO \quad = \quad TDFO \quad \times \quad FECDM \quad . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; . \; (5.2)$$

$TDFEO$ specific for *H. contortus* and *C. curticei* were calculated on the basis of third stage larvae percentages of the respective species determined in the cultures as described in Heckendorn et al. (2006). On day 28, immediately after slaughter, the abomasa and small intestines were ligated, opened and washed thoroughly in order to collect the luminal contents. Adult worm counts and sex identification were performed in a 10 % aliquot.

5.2.8 Statistical analysis

FECDM, TDFEO and worm burden were analyzed at the end of the experiment by means of generalized linear models (GLM) under the assumption of negative binomial distributed residuals. For FECDM and TDFEO analogous models were fitted at the end of the CT-feeding period. For the analysis of aggregated data (e.g. worm burden), such

GLMs have the advantage that they allow untransformed data to be analysed and that statistical tests of significance for the parameters of the models have a higher statistical power and a reduced risk of type I and type II errors compared to corresponding tests with log-transformed data and models that assume normally distributed residuals (Wilson et al., 1996; Torgerson et al., 2005). Fodder intake, faecal output and animal live weight were analyzed in 'normal' linear regression models. Both, GLMs and normal regression models contained parameters for the different blocks and parameters for the different feeds. As the design matrices of all models were dummy-coded and the control fodder set as a reference category, tests of significance for each of the three fodder parameters corresponded to testing whether there are differences in the response variable between any of the groups that received CT-containing forages and the control fed group (ryegrass : lucerne).

In order to establish a dose-response relationship, the relative reductions in TDFEO between the start and the end of the CT-feeding period were regressed as linear functions of the individual cumulative CT-intake. No statistical analyses were performed on the agronomical-, the feed analytical- and the PCV data as these measurements aimed only at describing the forages and at monitoring the health status of the animals, respectively. All data were analysed using STATA 9.0 (StataCorp LP, 4905 Lakeway Drive, Texas 77845, USA) software.

5.3 Results

5.3.1 Botanical feed analyses

An overview of the botanical composition of the different feeds during the CT-feeding period is provided in figure 5.1 on the facing page. The control fodder had a ryegrass : lucerne ratio of 5 : 4 and the harvested samples contained over 90 % DM of sown species. In the harvests of the chicory, birdsfoot trefoil and sainfoin swards the relative contributions of these species to the harvest were 84, 68 and 61 % DM respectively, when averaged over the whole CT-feeding period. Especially for sainfoin it is important to note that the relative contribution of that species in the harvest was not constant but increased from 46 to 74 % within the 17 days CT-feeding period. In all stands, unsown, competing species were mainly herbs such as Taraxacum officinale or legumes such as Trifolium repens.

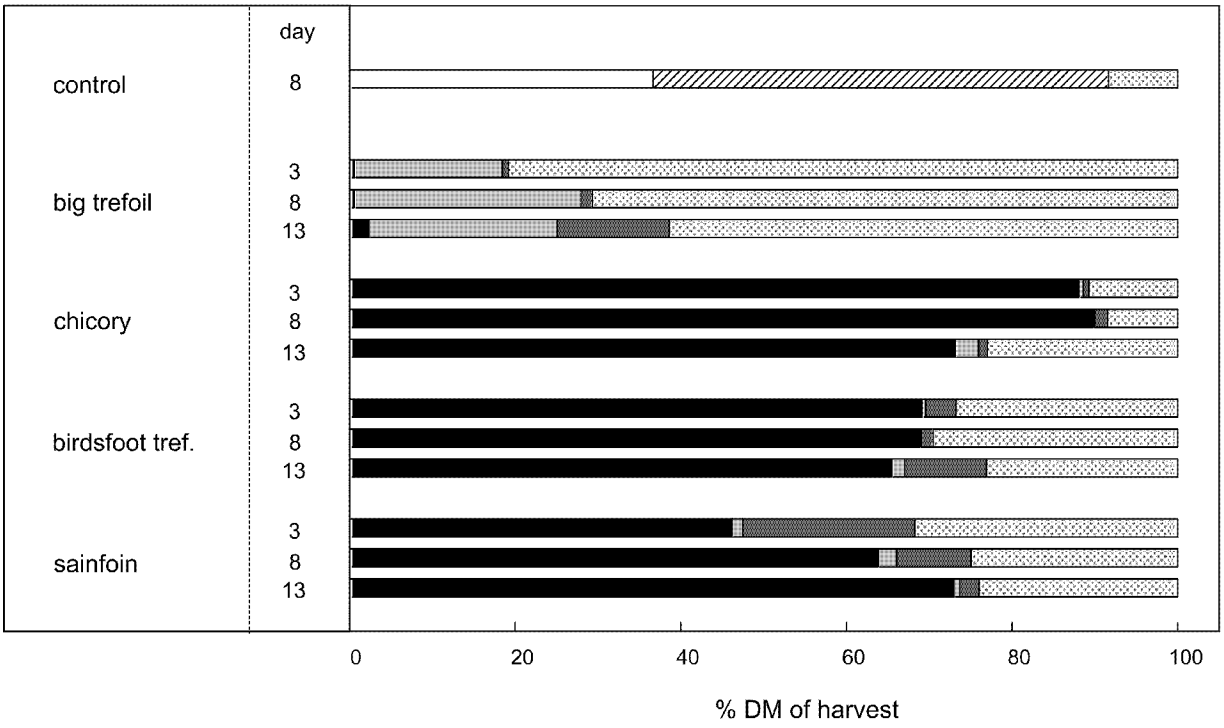

Figure 5.1: *Botanical composition of the harvests during the condensed tannin (CT) feeding period on days 3, 8 and 13 as functional groups. Sown lucerne (white), sown ryegrass (hatched), sown CT-containing forage (black), unsown legumes (light grey), unsown grasses (dark grey) and unsown herbs (dotted).*

5.3.2 Physical and chemical feed analyses

Physical and chemical key values of the different feeds are shown in table 5.1 on the next page. Dry matter contents of all CT-forages were relatively low and in vitro digestibility high compared to the control feed. Both differences were most pronounced for chicory where dry matter content was only half of that of the control forage but in vitro digestibility was 20 % higher. This will be important for the interpretation of faecal egg count data in the later text. Averaged over the whole CT-feeding period, sainfoin (26 g CT kg^{-1} DM) and birdsfoot trefoil (15 g CT kg^{-1} DM) had higher contents of condensed tannins than chicory or the grass : lucerne mixture (both values < 5 g kg^{-1} DM). In the case of sainfoin fodder, the CT content was not stable but increased according to its increasing biomass proportion in the harvest from 19 g CT kg^{-1} DM in the beginning to 34 g CT kg^{-1} DM at the end of the CT-feeding period (Fig. 5.1).

Table 5.1: *Mean dry matter content (DM), absorbable protein at the duodenum (APD), net energy (NE), in vitro organic matter digestibility (OMD) and condensed tannin (CT) concentration of the experimental feeds.*

		Control	Chicory	Birdsfoot trefoil	Sainfoin
DM	(g kg^{-1} fresh matter)	248	117	187	196
APD	(g kg^{-1} DM)	85	92	98	93
NE	(MJ kg^{-1} DM)	4.5	6.3	5.2	4.9
OMD	(g kg^{-1} DM)	558	677	601	579
CT	(g kg^{-1} DM)	0.2	3.1	15.2	26.1

5.3.3 Live weight, feed intake and faecal output

At the beginning of the CT feeding period, the average animal live weight was 22.4 0.6 kg (mean SE). During the CT-feeding period, only animals of the sainfoin group consumed dry matter amounts comparable (930 $\pm$ 45 g day^{-1}) to those consumed by the control fed animals (1050 $\pm$ 60 g day^{-1}; Fig. 5.2 on the next page). Dry matter intake of lambs of the birdsfoot trefoil and the chicory groups was significantly lower compared to control fed lambs (27 % and 52 %, respectively, both $P < 0.01$). Daily live weight gains were generally low and in birdsfoot trefoil fed animals were comparable to those achieved by control fed animals (80 $\pm$ 30 g day^{-1} and 70 $\pm$ 40 g day^{-1}, respectively). Highest daily weight gains where achieved in the sainfoin group (120 $\pm$ 20 g day^{-1}) whereas for chicory fed animals no weight gain was recorded within the 17 days CT-feeding period. Although the differences in daily live weight gain between feeds were remarkable, this was not reflected in significant differences in live weight between the feeding groups at the end of the study (data not shown); probably because of compensatory fodder intake during the 11 days of control feeding.

Due to the differences in dry matter intake and digestibility between the different diets, faecal output was significantly affected by the forage treatment ($P < 0.001$). Compared to the control animals (408 $\pm$ 25 g), the overall mean faecal output during the CT-feeding period was reduced by 293 $\pm$ 26 g and 142 $\pm$ 26 g faecal dry matter for chicory and birdsfoot trefoil (both $P < 0.01$), respectively, and by 56 $\pm$ 27 g ($P < 0.05$) in the sainfoin group.

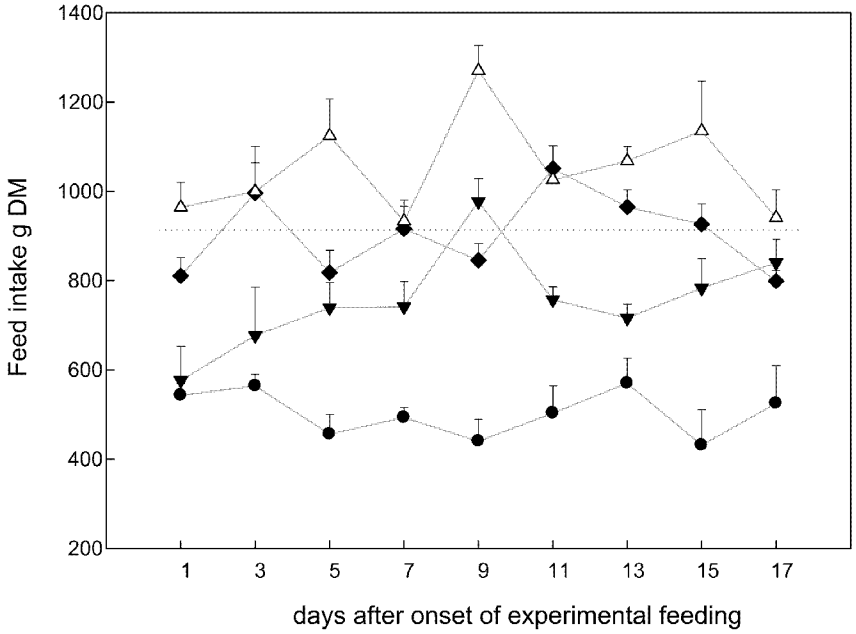

Figure 5.2: *Mean dry matter (DM) intake of lambs consuming ryegrass : lucerne (open triangles), chicory (closed circles), birdsfoot trefoil (closed triangles) or sainfoin (closed diamonds) during the condensed tannin (CT) feeding period. Bars indicate SE's of the means. The dotted line symbolises the expected DM intake of a lamb with a mean live weight of 22 kg (equals the mean live weight of lambs included in the experiment) and assuming moderate live weight gain of 200g day^{-1} as given in RAP (1999).*

5.3.4 Faecal egg counts (FECDM) and total daily faecal egg output (TDFEO)

Regular recordings of FECDM during the entire experiment are presented for each individual lamb in figure 5.3 on page 115. Infection intensities were already highly variable at the beginning of the experiment. By allocating the animals to blocks according to their initial FEC (i.e. 24 days p.i.), it was possible to obtain four comparable groups and to control this nuisance parameter when analysing the effect of the different forages at the end of the study. Average FECDM of the control fed group were fairly stable at about 15'000 eggs per gram of dried faeces from the beginning of the feeding experiment until the animals were slaughtered. For the chicory fed-group, FECDM increased markedly during an early phase of the CT-feeding period, partly because of a concentration effect due to the reduced fodder throughput. One animal of this group had to be removed from the study after 11 days of experimental feeding (i.e. 38 days p.i.) because PCV fell below a value of 15 (exclusion criterion). After this initial increase, FECDM started

to normalize in chicory fed animals five days after the start of the CT-feeding and in the end of the CT-feeding period, FECDM was non-significantly reduced by 44 % compared to control fed animals (Tab. 5.2 on page 116). This reduction persisted in the subsequent 11 days period of control feeding. Birdsfoot trefoil and sainfoin feeding decreased FECDM instantly and rapidly, arriving at FECDM reductions of 47 % ($P = 0.11$) and 57 % ($P < 0.05$) at the end of the CT-feeding period compared to control, respectively (Fig. 5.3, Tab. 5.2). For both feeds, FECDM remained low compared to the control after CT-feeding ceased. The final FECDM of birdsfoot trefoil or sainfoin fed animals, just before the slaughter of the sheep, were reduced by 65 % ($P < 0.01$) and 32 % ($P = 0.24$) compared to controls, respectively (Tab. 5.2). All CT-containing forages used in this experiment significantly reduced the FECDM specific to *H. contortus*, but none of them reduced the FECDM specific to *C. curticei* (Tab. 5.2).

Compared to the control group and consistent with FECDM, all CT fed groups had significantly reduced TDFEOs at the end of the CT-feeding period (all CT-fed groups: $P < 0.01$). TDFEO was reduced by 81, 53 and 58 % compared to controls for chicory, birdsfoot trefoil and sainfoin, respectively. Also in line with FECDM was the observation that the reduction of faecal egg output was solely due to a reduction of *H. contortus* specific TDFEO while *C. curticei* was apparently unaffected.

5.3.5 Worm burden

The worm burden of *H. contortus* was consistently but not significantly lowered in all CT-fed groups when compared to the control and this reduction was more pronounced for female than for male worms (Tab. 5.2 on page 116). In line with FECDM and TDFEO, no reductions of the *C. curticei* worm burden were observed for any feeding group.

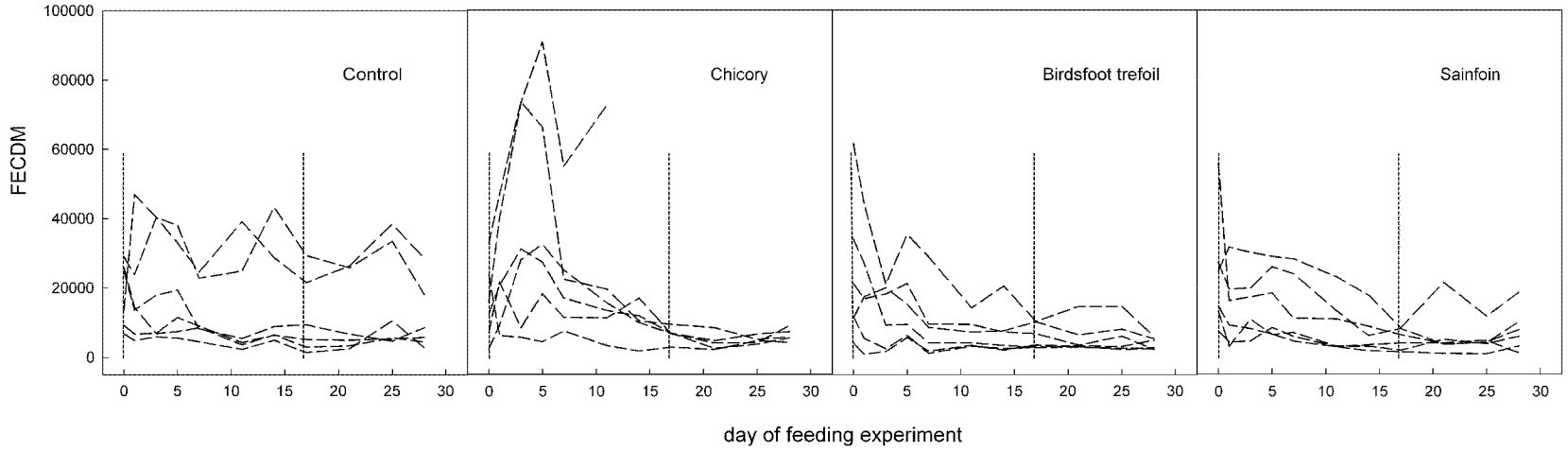

Figure 5.3: *Faecal egg counts per gram dry faeces (FECDM) during the entire experimental period (N / group = 6). The dotted lines indicate the beginning and the end of the CT-feeding period. Summary statistics and statistical test results at the end of the CT-feeding period and at the end of the experiment are provided in table 5.2. Note that in the chicory group one lamb had to be removed from the experiment on day 11.*

Table 5.2: *Mean faecal egg count per gram dry faeces (FECDM) and total daily egg output (TDFEO) values at the end of the condensed tannin (CT) feeding period and mean FECDM and mean worm counts at the end of the experiment of lambs in the control, chicory, birdsfoot trefoil and the sainfoin group. Percentages indicate the difference in means with respect to the control.*

	Control	***Chicory*†**			***Birdsfoot trefoil***			***Sainfoin***		
	Count	Count	Δ %	P	Count	Δ %	P	Count	Δ%	P
END OF CT FEEDING (DAY 17)										
FECDM										
Haemonchus	9418	2875	-69	**	3910	-58	*	3595	-62	*
Cooperia	2209	3659	66	n.s.	2199	-1	n.s.	1398	-36	n.s.
Total	11627	6534	-44	n.s.	6109	-47	n.s.	4993	-57	*
TDFEO (10^{-5})										
Haemonchus	29.8	3.1	-89	**	10.9	-63	*	11.0	-63	*
Cooperia	6.8	3.9	-43	n.s.	6.1	-11	n.s.	4.3	-38	n.s.
Total	36.6	7.0	-81	**	17.1	-53	**	15.3	-58	**
END OF EXPERIMENT (DAY 28)										
FECDM										
	11627	6367	-44	n.s.	3924	-65	**	7737	-32	n.s.
Wornburden										
Haemonchus ♀	285	168	-41	n.s.	120	-60	n.s.	148	-48	n.s.
Haemonchus ♂	255	292	15	n.s.	155	-39	n.s.	202	-21	n.s.
Total	540	460	-15	n.s.	275	-49	n.s.	350	-35	n.s.
Cooperia ♀	1412	1530	8	n.s.	1775	26	n.s.	1645	17	n.s.
Cooperia ♂	2215	2370	7	n.s.	2527	14	n.s.	2462	10	n.s.
Total	3627	3900	8	n.s.	4302	19	n.s.	4107	13	n.s.

Significance of statistical tests: n.s. not significant, * $P < 0.05$, ** $P < 0.01$

†Note that for chicory means and relative reductions of parasitological measurements were calculated without the lamb removed from the experiment at day 11 of the study

5.4 Discussion

5.4.1 Are differences of CT-action against GIN related to the host organ?

Our results suggest that all investigated tanniferous forage plants were active against *H. contortus* but none against *C. curticei*. Concerning the controlled administration of fresh CT-containing fodder plants, this study for the first time demonstrated anthelmintic effects towards *H. contortus*, one of the most important GIN worldwide. Compared to the control, faecal egg counts (FECDM) and total daily faecal egg outputs (TDFEOs) specific to *H. contortus* were reduced consistently in all bioactive forage groups after 17 days of CT-feeding. These results were in accordance with tendencies of reduced *H. contortus* worm recoveries at the end of the experiment (chicory 15 %, birdsfoot trefoil 49 % and sainfoin 35 % reduction). Surprisingly and interestingly our data suggest that CT affected the female worms more severely than the male worms (Tab. 5.2 on the preceding page). As the mode of CT-action is unknown, it is difficult to plausibly explain this apparent sex bias. It is, however, a phenomenon that was observed consistently in all tested forages and also in a previous experiment (Heckendorn et al., 2006).

In contrast to *H. contortus* and irrespective of the CT-containing fodder used, no reductions of adult *C. curticei* worm burdens were found. This result is in line with data obtained by Niezen et al. (1998a) who found that 42 days of birdsfoot trefoil feeding did not reduce the established *C. curticei* worm burden when compared to the control. The CT-concentrations used by Niezen et al. (1998a) were comparable (20-30 g CTs kg^{-1} DM) to the concentrations used in our experiment (15.2 and 26.1 g CTs kg^{-1} DM for birdsfoot trefoil and sainfoin, respectively). However, experiments with higher concentrations of CTs suggested that *C. curticei* is not inherently resistant against condensed tannins. For example, FEC of *C. curticei* were significantly reduced when sainfoin silage or hay with CT-concentrations of 42 and 61 g CTs kg^{-1} DM, respectively, were fed for the same period of time as in the experiment presented here (Heckendorn et al., 2006). Additionally, in the above mentioned study of Niezen et al. (1998a), it was found that the *C. curticei* worm burden was significantly reduced by 37 % when sulla (*Hedysarium coronarium*) with a CT content of 80–100 g CTs kg^{-1} DM was administered. Thus, the available data suggest that higher concentrations of condensed tannins are needed for the treatment of *C. curticei* than for *H. contortus*. A comparison with recent results of in vivo experiments with tanniferous forages given to sheep infected with *T. circum-*

cincta and/or *T. colubriformis* suggests that also for this pair of GIN higher CT-levels are necessary to produce an antiparasitic effect on the intestinal species, whereas the abomasal species is susceptible to lower CT-concentrations Hoste et al. (2006). Taken together, it could be speculated that the antiparasitic effects of tanniferous forages generally is achieved at lower CT-levels in the abomasum than in the small intestine and therefore would be organ dependent rather than GIN species related. Although this pattern has repeatedly been observed for field grown tanniferous forages, results obtained in a study with quebracho (a CT-rich extract from the bark of the subtropical tree *Schinopsis spp.*) demonstrated that the abomasal nematode *H. contortus* was unaffected by repeated drenches with high doses of quebracho (80 g CTs kg^{-1} DM) for 3 days (Athanasiadou et al., 2001). The apparent lack of effect on *H. contortus* in the study by (Athanasiadou et al., 2001) could be the result of the short exposure of the worms to quebracho (i.e. 3 days) compared to studies with forage CTs. Furthermore, discrepancies in anthelmintic effect due to the differences in the administered formulation (i.e. fodder CTs actively extracted by the host, quebracho CTs administered as a drench) cannot be excluded.

5.4.2 Plant specific anthelmintic activity of condensed tannins

Although all feeds containing CTs are highly palatable to sheep (Lüscher et al., 2005), feed intake in the experiment was generally low. This was most probably related to the combined effect of the parasite infections and the high temperatures in summer 2004. Nevertheless, all tanniferous forage plant species investigated in this study showed antiparasitic effects with respect to at least some of the relevant parasitological measurements. However, the apparent anthelmintic activity of chicory (*Cichorium intybus*) seems puzzling considering that the CT-concentration of that fodder was very low (3 g CTs kg^{-1} DM). Yet, anthelmintic effects have repeatedly been demonstrated for chicory (Marley et al., 2003; Thamsborg et al., 2004; Tzamaloukas et al., 2005). Possibly chicory owes its antiparasitic effect not only to CTs but also, or alternatively, to the high content of phenolic metabolites other than CTs (Tzamaloukas et al., 2005) or to its content of sesquiterpene lactones for which anthelmintic effects have been shown in vitro (Molan et al., 2003). Based on the results of our experiment, however, the feeding of young lambs with chicory cannot be recommended. We could not detect any live weight gain during the 17 days CT-feeding in the chicory group possibly due to a limitation of dry matter intake by the small rumen size and the high water content of this forage (see also section 4.4.). Furthermore, the chicory group was the only group from which an animal had to be excluded because of haemonchosis (PCV < 15).

Birdsfoot trefoil (*Lotus corniculatus*) reduced adult *H. contortus* parasite burden, faecal egg counts and total daily faecal egg output consistently by about 50 % compared to the control fed animals. Other studies examining the anthelmintic effect of freshly administered birdsfoot trefoil exclusively worked with natural GIN infections and it is therefore difficult to compare these results with the findings of our study. The majority of these experiments found no effect of birdsfoot trefoil feeding on GIN (Niezen et al., 1998a; Bernes et al., 2000; Hoskin et al., 2000). Only in one experiment, feeding this fodder to growing lambs was associated with a reduced abomasal worm burden and reduced FEC, which is in accordance with our findings (Marley et al., 2003).

Sainfoin (*Onobrychis viciifolia*) had the highest content of condensed tannins of all tested plants and showed similar activity against GIN as birdsfoot trefoil while allowing a higher daily weight gain than the one achieved by control fed animals. Antiparasitic effects of sainfoin condensed tannins (and also of flavonol glycosides) have been confirmed in vitro (Barrau et al., 2005) and in vivo (Thamsborg et al., 2004). Thamsborg et al. (2004) found 80 % FEC reduction and 35 % reduced adult *Teladorsagia* worm numbers in lambs co-infected with *Trichostrongylus vitrinus* after 3 weeks of sainfoin grazing when compared to a grass-clover fed control. Recent studies suggested that the anthelmintic effect of sainfoin is also preserved in sainfoin hay and silage (Paolini et al., 2003b, 2005a; Heckendorn et al., 2006). Preservation of sainfoin, particularly in the form of silage, seems very promising: It can be used for the control of GIN independent of the season and more of the forage plants' leaflets, which are particularly rich in CTs, are retained than during the hay-making process (Häring et al., 2007; Heckendorn et al., 2006).

5.4.3 Is there an in vivo dose-response relationship?

It is widely accepted that the antiparasitic effect associated with the feeding of sulla, sainfoin, bigfoot trefoil and birdsfoot trefoil can to some extent be attributed to their content of condensed tannins. In part, this view is supported by in vivo studies with quebracho and by in vitro studies with various sources of tannin (Athanasiadou et al., 2000; Molan et al., 2000a,b, 2003). However, causality between the antiparasitic effect associated with the feeding of any of the above mentioned bioactive forages and their tannin content has never been demonstrated in an in vivo experiment. Most in vivo experiments have been conducted by comparing the effect of a tanniferous forage to

that of a non-tanniferous but also in many other respects different control forage. Confounding with and interference of other primary and secondary metabolites cannot have been ruled out therefore. The only experiment known to us that used the same forage - big trefoil (*Lotus pedunculatus*) - both as treatment and as control (but in the control polyethylene glycol (PEG) was used in an attempt to inactivate CTs) failed to demonstrate that condensed tannins are responsible for the anthelmintic activity (Niezen et al., 1998b). An in vivo dose response curve for any forage plant and any parasite species would help to substantiate the role of condensed tannins as antiparasitic compounds. As in this study detailed data on fodder intake and on the CT-content of each forage were collected during the CT-feeding period, we related the CT-dose defined by the cumulative CT-intake over the 17 days CT-feeding period to the relative reduction in TDFEO within the same period (Fig. 5.4 on the facing page). Since we did not find any effect on *C. curticei*, data are shown for *H. contortus* only. The values for chicory fed lambs were excluded from calculation and are not present in the diagram because the anthelmintic effect of this fodder is probably associated with other secondary plant metabolites (see discussion above). For the remaining lambs, there was a slight trend across forage species for an increased antiparasitic effect with higher CT-doses. Particularly conspicuous however, are the strong relative reductions in TDFEOs in four lambs that received non-CT containing control fodder (lower left corner of Fig. 5.4). Interestingly, these were four sheep that had a comparatively low infection at the beginning of the CT-feeding period whereas the two strongly infected sheep in the control group still had strong infections at the end of the experiment. In contrast, the strongest relative reductions of TDFEO in birdsfoot trefoil and sainfoin fed animals were due to a reduction of TDFEO in sheep with the strongest infection at the start of the CT-feeding period. Overall, evidence indicates that a relationship between the cumulative CT-dose and the relative reduction in TDFEO for *H. contortus* might exist. Further dosing trials specifically addressing the question of dose-response relationships are necessary in order to get further insights to dose effects of individual sources of CTs towards GIN.

5.4.4 Reversibility of parasitological effect

The most commonly reported effect of tanniferous fodder plants is a substantially decreased faecal egg count (Hoste et al., 2006). Although this effect can be of great importance with respect to pasture contamination it is not necessarily a proof of a reduced worm burden or an improved health condition of the host. In fact, studies with goats found that reductions in FEC were essentially related to reductions in female worm fecundity with a potential of a renewed increase of FEC when the exposure to CTs is

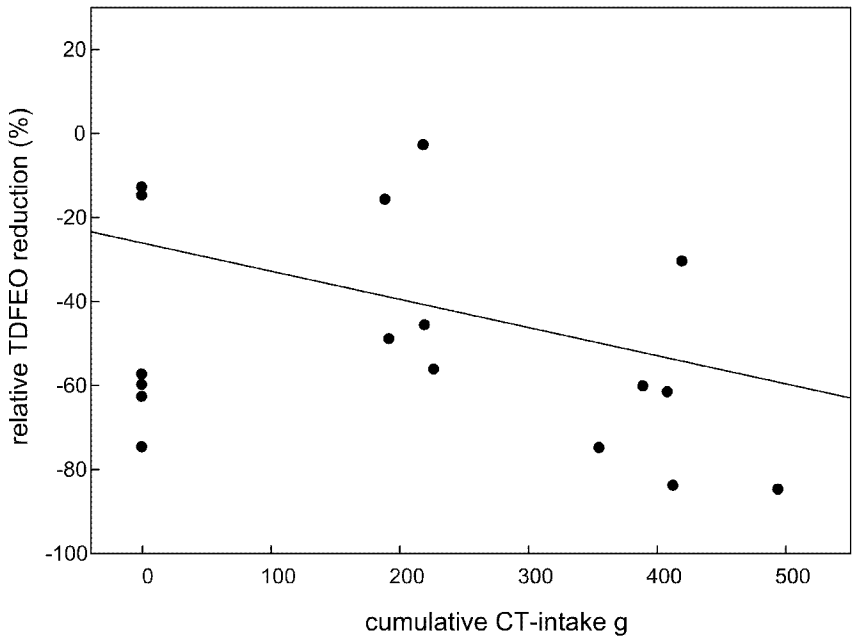

Figure 5.4: *Relative total daily egg output (TDFEO) reduction related to cumulative CT-intake during the 17 days CT-feeding period of control, birdsfoot trefoil and sainfoin fed animals. One value (X: 145, Y:133) is not shown in the graph but is included in the calculation of the regression line.*

withheld (Paolini et al., 2005a,b). Similarly, Min et al. (2004) and Lange et al. (2006) found only temporarily reduced *H. contortus* FEC after *Sericea lespedeza* (*Lespedeza cuneata*) hay feeding. In these studies FEC recovered to before treatment levels after the cessation of CT-feeding. In other experiments conducted mainly with sheep and relatively high concentrations of CTs, the observed FEC reductions were a true consequence of a reduced worm burden (Scales et al., 1994; Niezen et al., 1995, 1998b, 2002; Thamsborg et al., 2004; Heckendorn et al., 2006). This is in accordance with the results of the present study which was specifically designed to detect reversibility effects of FECDM. We found that predominantly the reduced female *H. contortus* worm burdens in the CT-fed groups were responsible for the FECDM reductions. In our experiment, it is unlikely that any indirect mechanism of CTs against *H. contortus* such as an enhanced immune response (resistance) mediated by improved protein availability could have been responsible for the observed anthelmintic effects as protective immunity against *H. contortus* is developed in lambs only after the age of 6 months (Urquhart et al., 1966a,b) which was not the case for our animals. Hence, CT-feeding was associated with an animal health gain in terms of a lower worm burden due to a direct detrimental effect of CTs against adult *H. contortus* (mainly against female worms)

and also with a reduced pasture contamination because of the sustainable reduced egg output - at least for the period examined in this experiment.

5.4.5 Interpretation of faecal egg counts can be ambiguous in feeding trials

There were major differences with regard to fodder intake between the four feeding groups. Most strikingly, dry matter intake of chicory fed animals was only about half compared to control fed lambs. We believe that rumen size has limited the fresh weight intake of the young lambs. Subsequently, the high water content and the comparatively elevated dry matter digestibility of chicory reduced the faecal output in this group compared to the control. Averaged over the CT-feeding period, faecal output of chicory fed animals was 72 % lower than of control fed ones. Of course, this was not without consequence with regard to faecal egg counts. Under the assumption of an equal worm burden and an equal fecundity of female worms, FECDM would have been 3.6-fold in chicory fed lambs compared to the control. Thus, the initial increase in FECDM at the start of the CT-feeding period in the chicory group can fully be attributed to the reduced fodder throughput. The fact that FEC is sensitive to dry matter content and digestibility of the feeds and also to the actual feed intake, casts some doubts on the usefulness of this parameter in feeding trials. Whenever possible, total daily faecal egg output (TDFEO) should be preferred to FEC when the effect of different fodders is to be compared.

5.4.6 Conclusion

In this study all investigated tanniferous forage plants were active against *H. contortus* but none against *C. curticei*. It is proposed that in the case of birdsfoot trefoil and sainfoin, anthelmintic effects were principally related to the action of CTs, whereas for chicory other secondary plant metabolites such as sesquiterpene lactones need to be considered. *C. curticei* does not seem inherently resistant to CTs but probably higher levels of these compounds are needed to produce anthelmintic effects. Together with the available data from other studies our data suggest that the anthelmintic effect of CT-containing fodder is to some extent dependent on the location of the parasites in the gastro-intestinal tract. The sustainable reduction of faecal egg counts as well as the lowered *H. contortus* burden at the end of the experiment suggested that the anthelmintic activity of CTs has directly affected the adult parasites of this species.

Limitations of standard faecal egg count as an indirect measure for the severity of a parasite infection in feeding trials was demonstrated. Whenever possible, total daily egg output should be preferred to FEC because FEC is insensitive to differences in fodder throughput between feeding groups. Overall, birdsfoot trefoil and in particular sainfoin seemed promising in contributing to an alternative, integrated control strategy against GIN not only by mitigating parasite related health disturbances of the host but also by a sustained reduction of pasture contamination by reduced egg output.

5.5 Acknowledgements

The financial support from the Swiss Federal Office for Agriculture strongly contributed to the realization of this work and is highly acknowledged. Special thanks go to Erika Perler, Zivile Amsler, Ilse Krenmayr, Lucia Kohler and Kathrin Bühler for irreplaceable backup in the lab and in the field. In vitro digestibilities where measured at the Research Station Agroscope Reckenholz - Tanikon ART and all nutritive analysis where done at the Research Station Agroscope Liebefeld-Posieux. The help of both is highly acknowledged.

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ScienceDirect
Veterinary Parasitology 142 (2006) 293–300
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veterinary parasitology
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Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs

Felix Heckendorn [a,b,*], Dieter Adrian Häring [d], Veronika Maurer [a], Jakob Zinsstag [c], Wolfgang Langhans [b], Hubertus Hertzberg [a,c]

[a] *Department of Veterinary Parasitology, Research Institute of Organic Agriculture, Ackerstrasse, CH-5070 Frick, Switzerland*
[b] *Physiology and Animal Husbandry, Institute of Animal Sciences, ETH Zurich, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland*
[c] *Swiss Tropical Institute, Socinstrasse 57, P.O. Box, CH-4002 Basel, Switzerland*
[d] *Agroscope FAL Reckenholz, Swiss Federal Research Station for Agroecology and*

Chapter 6

Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs

HECKENDORN F.[1] HÄRING D.A.[23], MAURER M.[1]ZINSSTAG J.[4]LANGHANS W.[5], HUBERTUS HERTZBERG[16]

Published in *Veterinary Parasitology* 142: pp. 293–300, 2006.

Just like the last one, this chapter is the result of a cooperation between the module *Plant Sciences* and the module *Parasitology* within the *Tannin-Project*. It investigates the antiparasitic effect of tanniferous hay and silage against parasitic nematodes in sheep. The author of this thesis conducted the chemical feed analysis and was involved in the thinking and writing process.

[1]Research Institute of Organic Agriculture, Frick
[2]Agroscope Reckenholz-Tänikon Research Station ART, Zurich
[3]Institute of Plant Sciences, ETH Zurich
[4]Swiss Tropical Institute, Basel
[5]Institute of Animal Sciences, ETH Zurich
[6]Institute of Parasitology, University of Zurich

6.1 Abstract

The objective of the study was to examine the effect of dried and ensiled sainfoin (*Onobrychis viciifolia*) on established populations of *Haemonchus contortus* (abomasum) and *Cooperia curticei* (small intestine) in lambs under controlled conditions. Twenty-four parasite naïve lambs were inoculated with a single dose of infective larvae of these parasites 28 days prior to the start of the feeding experiment. Twenty-four days post-infection, 4 days prior to the start of the feeding experiment, animals were allocated to four groups according to egg excretion, liveweight and sex. Groups A and B received sainfoin hay and control hay, respectively, for 16 days. Groups C and D were fed on sainfoin silage or control silage for the same period. Feeds were offered ad libitum and on the basis of daily refusals were supplemented with concentrate in order to make them isoproteic and isoenergetic. Individual faecal egg counts on a dry matter basis (FECDM) were performed every 3–4 days and faecal cultures and packed cell volume (PCV) measurements were done weekly. After 16 days of experimental feeding, all animals were slaughtered and adult wormpopulations were determined.

The consumption of conserved sainfoin was associated with a reduction of adult *H. contortus* (47 % in the case of hay, $P < 0.05$; 49 % in the case of silage, $P = 0.075$) but had little effect on adult *Cooperia curticei*. Compared to the controls, *H. contortus* specific FECDM was reduced by 58 % ($P < 0.01$) in the sainfoin hay group and by 48 % ($P = 0.075$) in the sainfoin silage group. For both sainfoin feeds FECDM specific to *C. curticei* were significantly decreased when compared to the control feeds (hay 81 % and silage 74 %, both tests $P < 0.001$). Our data suggest that different mechanisms were responsible for the reduction in FECDM in response to feeding tanniferous fodder. For *H. contortus*, the decrease seemed to be due to a nematocidal effect towards adult *H. contortus*. In contrast for *Cooperia curticei*, the reduction in FECDM appeared to be a result of a reduced per capita fecundity. For both, hay and silage, an antiparasitic effect could be shown, offering promising perspectives for the use of conserved tanniferous fodder as a complementary control approach against GIN.

Keywords:

Sainfoin; tannins; hay; silage; gastrointestinal nematodes; sheep; control

6.2 Introduction

Nematode infections of the gastrointestinal tract represent a major constraint in sheep husbandry, resulting in significant production losses (Brunsdon & Vlassoff, 1982; Coop et al., 1985; Parkins & Holmes, 1989; Sykes, 1994). For almost 50 years, the control of these parasites has relied almost entirely on the repeated use of anthelmintics (Williams, 1997). There are however several factors highlighting the need to develop alternative approaches in gastrointestinal nematode (GIN) control. These include widespread anthelmintic resistance within worm populations (Jackson & Coop, 2000) and the concern of consumers for drug residues in animal products (Waller, 1999). One complementary approach to reduce the dependence on anthelmintics is the use of tanniferous plants to limit nematode infections. Controlled indoor and outdoor studies with sheep have shown that the consumption of tanniferous legume forages like sulla (*Hedysarium coronarium*), big trefoil (*Lotus pedunculatus*) or birdsfoot trefoil (*Lotus corniculatus*) were associated with negative effects on host parasitism (Niezen et al., 1995, 1998b; Kahn & Diaz-Hernandez, 1999; Min & Hart, 2002; Marley et al., 2003). In parasitized goats, promising results have recently been obtained with sainfoin (*Onobrychis viciifolia*) hay (Paolini et al., 2003b, 2005a; Hoste et al., 2005). These reports for the first time documented that the anti-parasitic effects were preserved when using a tanniferous legume in conserved form.

To our knowledge, however, no experimental work exists with ensiled tanniferous plant material, although this conservation procedure is often preferred by farmers in regions with moderately warm summer temperatures, which limit the hay production of several fodder plants. Furthermore, the use of conserved tanniferous plants (hay and silage) against GIN has never been evaluated in sheep. Given the extensive body of knowledge accumulated in the last two decades, it is surprising that only few reports have focused on *H. contortus* (Athanasiadou et al., 2001; Paolini et al., 2003a, 2005b) although this parasite is probably the most important sheep nematode world-wide. In addition to *H. contortus*, *Cooperia curticei* was included in the study as a widespread intestinal species with regional importance (Rehbein et al., 1996, 1998). The objectives of the current study were, to determine the effects of ensiled and dried sainfoin on established populations of *H. contortus* and *Cooperia curticei* in lambs and to assess the consequences of these two treatments on animal productivity.

6.3 Materials and methods

6.3.1 Animals

Twenty-four Swiss White Alpine $\times$ Swiss Black- Brown Mountain lambs (10 females and 14 males) were used in the study. They were reared in a common pen under conditions that minimized nematode infection. The lambs were 2.5–3-months old and had a mean live weight of 33.1 $\pm$ 0.1 kg at the start of the trial. All animals were treated with levamisole (Endex 8.75 %, 7.5 mg/kg body weight) to ensure helminth-free conditions.

6.3.2 Forage and feed constituents

Four different experimental feeds were used. Sainfoin hay and silage were produced in summer 2004 from sainfoin (cv. Visnovsky) monoculture swards located at the Swiss Federal Research Station ALP (Posieux, Canton of Fribourg, 660 m above sea level) and at the Research Institute of Organic Agriculture (FiBL, Canton of Aargau, 350 m above sea level). For hay production the fresh sainfoin plant material was artificially dried for 48 h at 30°C using a vented drying chamber. Silage units of approximately 35 kg were produced by pressing the cut sainfoin at approximately 35 % dry matter (DM) and enwrapped in commercial silage film. Ryegrass/clover hay and a maize-lucerne silage were used as control forages, respectively. At the beginning of the experiment in early 2005, CT concentrations of all feeds were measured according to the butanol-HCl method described in Terrill et al. (1992). Feed constituents were determined as described in RAP (1999).

6.3.3 Parasite isolates

Infective larvae of *H. contortus* and *Cooperia curticei* were cultured from monospecifically infected donor lambs according to standard procedures. Parasite isolates were kindly provided by Merial, Germany.

6.3.4 Experimental design and measurements

Twenty-eight days prior to the start of the feeding experiment all lambs were inoculated with a single dose of 7000 third stage larvae of *H. contortus* and 15,000 third stage

larvae of *Cooperia curticei*. On the basis of faecal egg counts, individual weight and sex on day 24 postinfection (p.i.), lambs were assigned to one of four experimental Groups A–D consisting of six animals each. Groups A and B consisted of two female and four male animals each and starting from day 28 p.i. received sainfoin or ryegrass hay, respectively, for 16 consecutive days. Animals of Groups C and D consisted of three male and three female animals each and were fed with either sainfoin silage or with maize-lucerne silage for the same period. Lambs were offered the different feed ad libitum. On the basis of refusals per group, nutrient contents of the feeds and live weight of the lambs, the feeds were daily adjusted with a commercial concentrate (UFA 763; UFA AG, CH-6210 Sursee) or soy meal, in order to make them isonitrogenous and isoenergetic.

During the CT-feeding period individual faecal samples were taken from the rectum every 3–4 days for faecal egg counts and faecal cultures were made weekly for each feeding group. Faecal dry matter content was determined in a 3 g sub-sample dried in a force draught oven at 105℃ for 16 h immediately after collection. Since from day 3 onwards the faecal dry matter in the sainfoin silage group (pooled means $\pm$ S.E.M. 31 $\pm$ 1.5 %; $P < 0.05$) was significantly elevated compared to all other feeding groups, faecal egg counts were expressed as the number of eggs per gram of dried faeces (FECDM). Every week individual live weights were recorded and blood samples were taken for packed cell volume (PCV) measurements. At day 45 post-infection, (p.i.) all animals were slaughtered. Immediately after death the abomasa and small intestines were separated, opened and washed in order to collect the luminal contents. Adult wormcounts and sex identificationwere performed in a 10 % aliquot.

6.3.5 Faecal samples and culture processing

Faecal samples were processed immediately for FEC (Schmidt, 1971). Pooled quantitative faecal cultures were prepared group-wise using a 2 g sub-sample of fresh faeces from every lamb (Larsen, personal communication). Prior to cultivation the faecal material was homogenized thoroughly in order to uniformly distribute the eggs and pre-culture FEC were performed. Per culture 12 g of faecal material was placed in a polystyrene container and incubated for 10 days at 20℃ under conditions that maximised humidity. Postculture, larvae were extracted for 24 h using a Baermann apparatus and transferred to Falcon tubes. After a 6 h storage period at 4℃ excess liquid was removed by siphoning and the concentrated larvae were transferred to tissue culture flasks and stored at 6℃ until processing. Total numbers of infective larvae were

counted in 500 ml aliquots (10 $\times$ 50 ml) and the mean counts extrapolated to the total culture volume. Furthermore, a total of 100 L3 larvae were differentiated within every culture. FECDM specific for *H. contortus* and *C. curticei* were calculated on the basis of L3 percentages of the respective species determined in the cultures, assuming equal development of the two GIN species (Borgsteede, personal communication). Per capita fecundity (PCF) was calculated separately for *H. contortus* and *Cooperia curticei* by dividing the species specific FECDM recorded at slaughter by the total numbers of female worms recovered.

6.3.6 Statistical analysis

All data were analysed using STATA 9.0 (Stata- Corp LP, 4905 Lakeway Drive, TX 77845, USA) software. Evidence of aggregated distributions for both FECDM and worm burden was confirmed. Aggregated data are defined as the variance being greater than the mean (Torgerson et al., 2005). For FECDM and worm burdens cross-sectional negative binomial regression models were therefore fitted separately for each point in time with the two parameters of the model being the arithmetic mean and the negative binomial constant. The mean egg count or worm burden and the 95 % negative binomial confidence intervals were estimated by maximum likelihood techniques. Comparisons were made between the (i) sainfoin hay and the control hay group and (ii) the sainfoin silage and the control silage group. Equivalent comparisons were done for the worm burdens. Per capita fecundity, PCVand live weight were analysed using *t*-tests.

6.4 Results

6.4.1 Nutritional contents and condensed tannin concentrations

Net energy contents of the feeds were comparable within the hay groups (sainfoin 5.1 MJ/kg DM, control 5.0 MJ/kgDM) and the silage groups (5.9 MJ/kg in both groups). Compared to control hay, sainfoin hay had a higher protein content (77 g/kg versus 93 g/kg DM), whereas the two silage groups where essentially similar (70 g/kg DM both). Sainfoin hay had a higher CT content than sainfoin silage (mean $\pm$ S.E.M., 6.12 $\pm$ 0.48 % DM and 4.19 $\pm$ 0.87 % DM). The CT-concentrations measured in the two control feeds were very low (mean $\pm$ S.E.M., hay 0.13 $\pm$ 0.01 % DM, silage 0.07 $\pm$ 0.03 % DM)

6.4.2 Consumption of feeds and live weight gain

There were no significant live weight differences between groups at the beginning of the study. All feeds were readily eaten by the lambs throughout the study period. Mean daily DM intakes per animal averaged over the entire experimental period were similar for all groups (approximately 1.2 kg DM d^{-1}, 6.1). No significant differences in daily weight gain were found between animals of the sainfoin silage group compared to those of the control silage group (mean $\pm$ S.E.M., 64 $\pm$ 27 and 84 $\pm$ 20 g). There was a trend of increased daily weight gain in the sainfoin hay group compared to the control hay group (mean $\pm$ S.E.M., 163 $\pm$ 20 and 96 $\pm$ 27 g; P = 0.07). However, no significant difference in live weight between the hay groups was present at the end of the study (sainfoin 35.8 $\pm$ 0.3 kg, control 34.8 $\pm$ 0.4 kg, P = 0.49).

Table 6.1: *Mean daily intake of dry matter (DM; mean $\pm$ S.E.M.), absorbable protein at the duodenum (APD; mean $\pm$ S.E.M.) and net energy (NE; mean $\pm$ S.E.M.) per animal of Groups A–D averaged over the 16-day study period.*

			DM (kg)	APD (g)	NE (MJ)
HAY FED					
	Group A	Sainfoin hay	0.98±0.02	88.29±1.23	5.34±0.18
		Concentrate UFA 763	0.20±0.01	22.76±1.39	1.71±1.39
		Total	**1.18**	**111.05**	**7.05**
	Group B	Control hay	1.27±0.01	98.94±0.73	6.46±0.05
		Concentrate UFA 763	0.06±0.01	6.35±0.53	0.51±0.04
		Total	**1.33**	**105.29**	**6.97**
SILAGE FED					
	Group C	Sainfoin silage	1.15±0.01	72.29±2.04	6.43±0.06
		Soy meal	0.06±0.01	18.03±1.05	0.48±0.04
		Total	**1.21**	**90.32**	**6.91**
	Group D	Control silage	1.09±0.02	77.12±1.07	6.37±0.06
		Soy meal	0.15±0.03	17.18±1.34	0.46±0.05
		Total	**1.24**	**94.30**	**6.83**

6.4.3 Faecal egg counts

For *H. contortus*, the reduction of specific FECDM associated to the feeding of sainfoin was substantiated in the course of the study (Figs 6.1 and 6.2 on the next page). After 16 days of consecutive experimental feeding *H. contortus* specific FECDM was reduced by 58 % ($P < 0.01$) in the hay group and by 48 % ($P = 0.075$) in the silage group compared to the respective controls. For C. curticei, already 3 days after experimental feeding had started, lambs fed with sainfoin hay or silage had a significantly reduced specific FECDM (both tests $P < 0.001$; Figs 6.3 on the facing page and 6.4 on page 138) compared to the controls. These differences remained stable until the end of the study (81 % reduction in sainfoin hay group ($p < 0.05$), 74 % reduction in sainfoin silage group ($p < 0.01$).

6.4.4 Worm burden and per capita fecundity

The total *H. contortus* burden was lowered by approximately 50 % by both sainfoin feeds and in the hay group this reduction was significant ($P < 0.05$; Table 2). The per capita fecundity of *H. contortus*, was not significantly different between both, hay (sainfoin 31.3 and control 26.5 eggs g^{-1} $female^{-1}$, $P = 0.60$) and silage groups (sain-

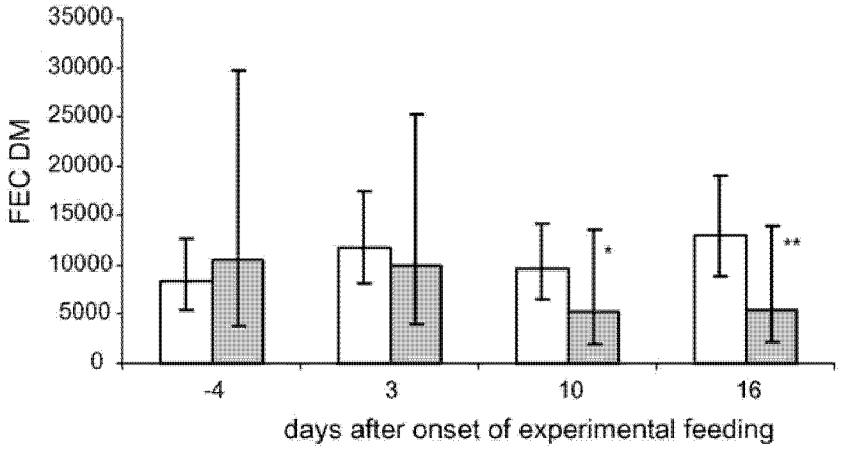

Figure 6.1: *Comparison of faecal egg counts based on faecal DM and specific to* H. contortus *in the group receiving control hay (open bars) or sainfoin hay (closed bars), respectively. Error bars indicate 95 % CI (maximum likelihood). Significance of statistical tests * $P < 0.05$; ** $P < 0.01$.*

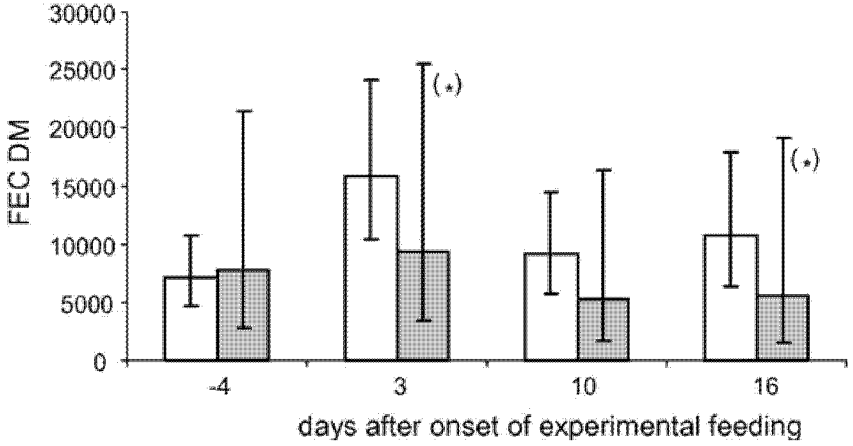

Figure 6.2: *Comparison of faecal egg counts based on faecal DM and specific to* H. contortus *in the groups receiving control silage (open bars) or sainfoin silage (closed bars), respectively. Error bars indicate 95 % CI (maximum likelihood). Significance of statistical tests * $P < 0.1$.*

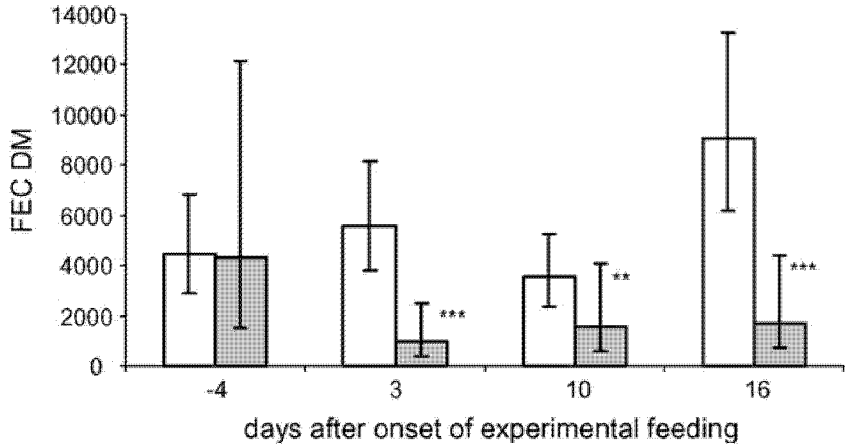

Figure 6.3: *Comparison of faecal egg counts (FEC) based on faecal DM and specific to* C. curticei *in the groups receiving control hay (open bars) or sainfoin hay (closed bars), respectively. Error bars indicate 95 % CI (maximum likelihood). Significance of statistical tests ** $P < 0.01$ *** $P < 0.001$.*

foin 18.7 and control 27.3, eggs g^{-1} $female^{-1}$ P = 0.51). Total *Cooperia curticei* worm counts were not substantially reduced by both experimental feeds compared to the control feeds (sainfoin hay 9 %, P = 0.58 and silage 14 %, P = 0.14, respectively). But, for *Cooperia curticei* a significantly lower per capita fecundity was found between the sainfoin hay and the control hay group (0.46 and 2.28 eggs g^{-1} $female^{-1}$, $P < 0.001$) and also between the sainfoin silage and the control silage group (0.48 and 1.68 eggs g^{-1}

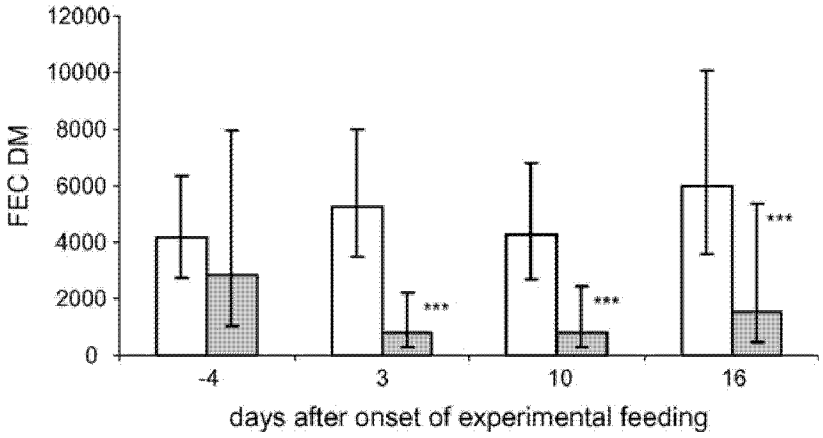

Figure 6.4: *Comparison of faecal egg counts (FEC) based on faecal DM and specific to* C. curticei *in the groups receiving control silage (open bars) or sainfoin silage (closed bars), respectively. Error bars indicate 95 % CI (maximum likelihood). Significance of statistical tests *** $P < 0.001$.*

female^{-1}; $P < 0.05$).

6.4.5 Packed cell volume

Until the end of the experiment there was no significant difference in PCV between the sainfoin silage group and the respective control group. PCV levels of the sainfoin hay group were significantly lower than in the control hay group at day 16 after onset of experimental feeding (mean $\pm$ S.E.M. 31.2 $\pm$ 0.7 % and 33.2 $\pm$ 0.7 %; $P < 0.05$) but were still in the physiological range (30–38 %).

6.5 Discussion

The main finding of this experiment is that by feeding sainfoin hay and silage for 16 consecutive days, the *H. contortus* worm burden was reduced by approximately 50 % compared to the corresponding controls. This is in contrast to other studies using condensed tannins, only documenting a decrease in FEC and fecundity of *H. contortus*. Athanasiadou et al. (2001) administered different doses of quebracho CT to *H. contortus* infected lambs and could not detect any difference in worm burden between drenched groups and control groups. Quebracho drenches were given only for 3 consecutive days, however, and the total study period was shorter than in our experiment

(9 days versus 16 days). Paolini et al. (2003a) tested quebracho CT given for 8 consecutive days at a concentration equivalent to 5 % of the dietary DM in goats artificially infected with *H. contortus*. No significant difference in worm burden was found after a total study period of 15 days when compared to a control group. It is known that the chemical composition of CT may alter its bioactive potential (Aerts et al., 1999). The results obtained with quebracho are therefore only of limited comparability to our study and must be interpreted carefully. A recent study evaluated the effect of repeated distribution of sainfoin hay in goats naturally infected with GIN, where the animals were fed with the hay for 7 consecutive days every month for a total period of 3 months (Paolini et al., 2005a). At the end of the study only fecundity of *H. contortus* was decreased, but no difference was seen in adult worm burden compared to a control group. The prolonged administration period of 16 consecutive days in our study might explain the increased effect on adult *H. contortus* worms. It is theoretically possible that a longer exposure of worms to CT is necessary in order to observe a nematocidal effect. This hypothesis is partly supported by a report focussing on another abomasal nematode (*Teladorsagia circumcincta*), where the adult worm burden was significantly reduced by 90 % when feeding sulla (another tanniferous legume) for a period of 42 days (Niezen et al., 1994).

In our work, no differences in per capita fecundity of *H. contortus* females were observed between the sainfoin and the control groups. This finding is in line with results of Athanasiadou et al. (2001), where per capita fecundity of this parasite was unaltered in a quebracho fed group of lambs compared to a control group. In a study with goats, it was found that the fecundity of *H. contortus* was significantly decreased when feeding sainfoin hay (Paolini et al., 2005a). In that study, however, fecundity was assessed directly by counting eggs in utero and the observed difference to our results might be related to the difference in the applied methodology.

Compared to results obtained with *H. contortus*, we found only moderate reductions in adult *Cooperia curticei* worm burden regarding both sainfoin feeds when compared to the respective control groups. This finding is in contrast to results obtained by Niezen et al. (1998a) who reported a significant lower *Cooperia curticei* worm burden in lambs feeding sulla for 42 days compared to a control group. Studies looking at the effect of CT on other intestinal nematode species reported contradictory results. Significant decreases in adult *Trichostrongylus colubriformis* burden have repeatedly been observed (Niezen et al., 1995; Athanasiadou et al., 2000b; Paolini et al., 2005a), but also negative results have been reported (Niezen et al., 1998b). Unfortunately, these studies are

of limited comparability because unequal experimental designs and different sources of CT were used. Concerning per capita fecundity, however, data from different experiments on intestinal nematodes seem to be much more coherent. In our experiment, the per capita fecundity of *Cooperia curticei* was significantly lowered by both sainfoin feeds. This finding is in accordance with studies on *T. colubriformis* where per capita fecundity was lower when quebracho CT was administered (Athanasiadou et al., 2000a, 2001).

The results of the present study suggest that the significant decrease in FECDM is driven by two different mechanisms for the investigated nematode species. For *H. contortus* the decrease in FECDM seems to be mainly associated with the reduced worm burden, whereas for *Cooperia curticei* the lower fecundity is suggested to be the relevant parameter in lowering FEC. Overall, the observation of decreased FEC is in agreement with work done by Paolini et al. (2003b, 2005a), where FEC was significantly reduced when sainfoin hay was administered to goats naturally infected with GIN.

In our experiment the *Cooperia curticei* FECDM in the sainfoin silage group was already 31 % ($P = 0.223$) lower compared to the control hay group at the beginning of the experiment. This initial imbalance arose out of the delay of culture results and the need of a stratified group allocation according to FEC, which we were forced to do on total FEC. Still, a strong decline of the *Cooperia curticei* specific FECDM was observable in the sainfoin silage group when compared to the control.

It is well documented that an increase in feed protein will improve the resistance of sheep to GIN (Coop & Holmes, 1996; Van Houtert & Sykes, 1996). Resistance effects mediated by an improved immune response were not expected within the chosen experimental period (i.e. 16 days) and due to the age of the animals used (Urquhart et al., 1966a,b). Nevertheless, feeds in our work were adjusted for protein and energy in order not to disguise possible CT-effects. With respect to PCV levels, a significant difference was only observed between the sainfoin hay and the control hay group. However, this difference was not of physiological relevance. A likely explanation for the overall observed equality of PCV levels between sainfoin and control groups is a delay in response of the parameter to *H. contortus* worm burden with respect to the short experimental period. Work done by Paolini et al. (2005a) showed a significant reduction of PCV levels in control hay fed goats compared to sainfoin hay fed ones only 70 days post-infection, thus highlighting the need of a prolonged experimental period in order to

observe any effect on this pathophysiological parameter.

Concerning preserved sainfoin, this study for the first time presents results pointing to a nematocidal effect towards *H. contortus*. The physiological basis of the underlying interactions is still unclear and remains to be elucidated. In regions with moderate climatic conditions, the production of soil dry sainfoin hay is problematic because the cut plant needs a short and hot drying phase in order not to lose the CT-containing leaves in the drying process. As an easily feasible conservation alternative, ensiled sainfoin was therefore produced for this experiment. Although CT-contents were slightly lower in sainfoin silage as in hay, the antiparasitic effect was also present when using this conservation method. Further studies using sainfoin silage must be performed, in order to determine its effect on other GIN species and to evaluate the acceptance of the strategy among farmers.

Overall, conservation of tanniferous fodder plants offers exciting opportunities with respect to centralized production, sale, storage and an extended administration independent of the season. However, its potential broader use should also be subjected to an analysis of its profitability.

6.6 Acknowledgments

The authors are grateful to the Swiss Federal Office for Agriculture (BLW) for financial support and to the Swiss Federal Research Station for Animal Production and Dairy Products (ALP) for carrying out the nutritive analysis. Paul Torgerson from the Institute of Parasitology (IPZ), Zurich helped in statistics and Lucia Kohler aided in worm isolation. Their contributions are highly acknowledged. We also thank Erika Perler, Ilse Krenmayr and Zivile Amsler for irreplaceable back-up in the lab and Pius Allemann and his team for excellent technical support.

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Chapter 7

General discussion

The previous chapters explored dynamics of tannin concentrations on different hierarchical levels (i.e. on the level of plant tissues in Chapter 2, on the level of plant organs and entire plants in Chapter 3 and on the level of harvestable biomass of plant communities in Chapter 4). Furthermore, in cooperation with the partners of the *Tannin-Project*, the suitability of various tanniferous forage plant species for cultivation and application against gastrointestinal nematodes in sheep was tested in Chapters 4, 5 and 6. The collective work in this thesis should improve our general understanding of the dynamics of condensed tannin concentrations and it provides essential basic knowledge for the practical application of tanniferous forage plants for the control of gastrointestinal parasites of ruminants.

7.1 Predicting concentrations of condensed tannins

So far, most investigations of phenotypic variations of concentrations of secondary metabolites in plants were either purely descriptive (Borreani et al., 2003; Wen et al., 2003; Gebrehiwot et al., 2002; Roberts et al., 1993) or based on the theoretical framework of the *plant defence theory* (e.g. Riipi et al., 2002; Carter et al., 1999; Lindroth et al., 1987). Between experiments, results were often conflicting and the predictive accuracy of existing models rather low. Therefore, the following text starts with a critical discussion of the usefulness of the plant defence theory for the prediction of condensed tannin concentrations in the harvestable aboveground biomass of tanniferous forage plants. Then the modelling approach outlined in Chapter 3 is further developed to include competition by non-tanniferous species and the importance of the different potentially influential factors for the determination of the tannin concentration of the harvest

of pure and mixed stands is discussed.

7.1.1 Doubts about the usefulness of the 'plant defence theory'

The collective work in this thesis implies that central assumptions of the *plant defence theory* are either wrong or at least less generally true than commonly assumed. Crucial to all plant defence hypotheses[1] are the following two assumptions:

1. Herbivory is a primary selective force for the production of secondary metabolites (Stamp, 2003).

2. Secondary metabolites exert a plant protective effect against their consumers (Stamp, 2003).

For example, Feeny (1976) argued that quantitative defences like condensed tannins slow the growth rate of herbivores and also subject them to higher rates of predation and parasitism. However, In Chapter 5 and 6 of this thesis, it was shown that sheep consuming *Lotus corniculatus* or *Onobrychis viciifolia* with moderate tannin concentrations (i.e. 15 and 26 g CT kg^{-1} DM, respectively) had similar or even higher growth rates and lower levels of parasitism than sheep fed with a non-tanniferous control forage. Moreover, our experiments showed that although condensed tannins can reduce the digestibility of a forage, tanniferous forage plants like *Lotus corniculatus* or *Onobrychis viciifolia* are not less digestible than a grass / legume mixture commonly fed to sheep (Chapter 5). Furthermore, there was no evidence that plant species with higher levels of condensed tannins are better protected from being eaten (by sheep) than those with lower levels of condensed tannins (Chapter 4). In fact, *Onobrychis viciifolia* which had the highest tannin concentrations was more palatable to wethers than any other of the tested forages including a non-tanniferous grass / legume mixture. Possibly, the expected negative relationship between the concentration of condensed tannins of different forage plants and the amount consumed by the wethers was masked by the otherwise different chemical composition of the tested plant species (Villalba et al., 2002). Overall, it is undisbuted that condensed tannins *can* have a plant protective effect (this has been shown many times), but their defensive activity is probably less general than assumed by the *plant defence theory* (Häring et al., 2007; Heil et al., 2002; Goverde et al., 2001; Bernays, 1981). Therefore, the assumption of a plant defensive effect of

[1] Some of the most influential plant defense hypotheses are summarized in the introduction of this thesis commencing on page 11.

secondary metabolites seems a rather weak starting point for a reliable prediction of phenotypic variation of condensed tannins.

Another important assumption of the *plant defence theory* is that the production of secondary metabolites is costly to the plant. For example, the growth-differentiation hypothesis (Herms & Mattson, 1992) – which is regarded as the most mature of the plant defence hypotheses (Stamp, 2003; Koricheva, 2002a) – predicts that costs arise to the plant because secondary metabolism diverts resources from growth. With regard to plant development, it is predicted that *when a large proportion of modules within a plant are undergoing growth simultaneously, secondary metabolism will be limited by lack of substrate* (Herms & Mattson, 1992). During plant development of *Onobrychis viciifolia* (Chapter 3), the tannin concentration in leaves increased linearly without a detectable delay during periods of strong growth. At the level of the entire plant, tannin concentration declined, but as a consequence of an accumulation of tannin-poor plant material (i.e. the production of stems and roots) rather than as a result of substrate limitation.

Moreover, the growth-differentiation hypothesis and the carbon-nutrient balance hypothesis argue that *moderate nutrient deficiency limits growth more than photosynthesis*; *hence, nutrient-deficient plants accumulate carbohydrates, increasing the C / N ratio within the plant*. Subsequently, the *carbohydrates accumulated in excess of growth requirements are allocated to C-based secondary metabolites* (Stamp, 2003; Herms & Mattson, 1992; Bryant et al., 1983). On first sight apparently in line with this prediction, Chapter 2 demonstrated a negative relationship between, either soil fertility, growth rate or the C / N ratio of the plant and concentrations of condensed tannins. The frequent retrieval of such negative correlations have been interpreted as evidence in favour of either the carbon-nutrient or the growth-differentiation balance hypotheses (Koricheva et al., 1998; Koricheva, 1999). However, also in Chapter 2, it was found that despite a large potential for accumulation of carbon in excess of growth demands and regardless the apparent recognition of a simulated insect attack (i.e. concentrations of condensed tannins increased in response to the application of insect saliva), the induction of the concentration of condensed tannins was not stronger at low as compared to high nutrient fertility. Given the fact that the strength of the induction of condensed tannins was unrelated to the nutrient status of the plant either suggests that costs for the production of condensed tannins are not strongly related to the nutrient status of the plant (e.g. carbon is always 'cheap'; Craine et al., 2003), or, it implies that tannin accumulation is somehow constrained at low levels of nutrient availability. Although carbohydrates often accumulate in excess of the growth demand when growth is moderately limited

by shortage of water, nutrient or temperature (Häring & Körner, 2004; Körner, 2003; Hoch & Körner, 2003; Hoch et al., 2002; Koricheva et al., 1998), surplus carbon does not necessarily feed into the production of C-based secondary metabolites[2].

These results indicate that the often found lower concentrations of condensed tannins at high as compared to low nutrient availability should be interpreted as a result of dilution due to the accumulation of non-tanniferous plant material at high levels of fertility, rather than that the high tannin concentrations at low nutrient availability are interpreted as 'extra-tannins' due to 'cheap' surplus carbon.

Overall, the *plant defence theory* provided an interesting theoretical framework for the prediction of the variation of secondary metabolites in plants. However, today it is clear that central assumptions of the *plant defence theory* are not beyond doubt or even wrong. These assumptions include the co-evolution between phytophagous insects and plants (Edwards, 1992), the generality of plant-protective effects of secondary metabolites (Chapter 4 of this thesis; Bernays, 1981), the physiological mass-action and assimilate-driven view of the regulation of secondary metabolism (Häring & Körner, 2004; Nitao et al., 2002; Hamilton et al., 2001) and the generality and importance of a trade-off between defence and growth (Chapter 1 of this thesis; Koricheva, 2002b). In contrast to Koricheva (2002a) and Stamp (2003), I consider the growth-differential balance hypothesis not as 'more comprehensive' or 'more mature' than the carbon-nutrient balance hypothesis but rather as an extension of the latter; I agree with the view of Berenbaum (1995) that *an all-encompassing theory might be biologically unrealistic*. In my opinion, a model for the reliable prediction of concentrations of secondary metabolites in specific (forage) plants should be free of assumptions with regard to the functionality of the compound; it should allow a flexible adaptation to our increasing knowledge of biosynthetic regulatory mechanisms and it should acknowledge dilution as an important driver of secondary metabolite concentrations.

7.1.2 An alternative approach

As outlined in the introduction in Chapter 1 and experimentally demonstrated in Chapters 3 and 4, mixing of plant parts with different concentrations of condensed tannins

[2]In fact, if assimilates accumulate but do not feed into the pool of secondary metabolites, counter to the predicted outcome, concentration of secondary metabolites decrease instead of increase (e.g. Häring & Körner, 2004).

and dilution caused by accumulation of non-tanniferous plant material can be crucial for the determination of concentrations of condensed tannins in plant material. In Chapter 3, it was shown that during plant development, the increasing proportion of low-tannin stems in the harvest of tanniferous forage plants neutralized the effect of the increasing tannin concentrations in leaves with regard to the tannin concentration of the harvestable aboveground biomass. Accordingly, a modelling approach for the prediction of tannin concentrations in tanniferous plant species has been suggested and briefly but successfully evaluated on data of *Onobrychis viciifolia* grown in another, independent experiment (Borreani et al., 2003).

In Chapter 4, however, it was shown that the seasonal dynamics of tanniferous, field-grown forage plants followed closely the relative contribution of the tanniferous forage plants to the total dry matter yield of the plot (Fig. 4.2 on page 89). In other words, the seasonal dynamic of the tannin concentration was reasonably well predictable when the tannin concentration of the tanniferous plant was assumed constant (and the leaf / stem ratio ignored) during the entire experiment and fluctuations of tannin concentrations simply attributed to fluctuations in biomass proportions of tanniferous and non-tanniferous plant species. Thus, with the tested plant species and under the given conditions in Chapter 4, competition between tanniferous and non-tanniferous plant species was the most important determinant of the tannin concentration of the harvest of the experimental fields. The model presented in Chapter 3 (Eqn. 3.4 on page 67) can easily be extended to include competition between species; in equation 3.4 on page 67 it was proposed that the tannin concentration of a single tanniferous plant species $T_{\mathsf{CT}}(t)$ can be written as:

$$T_{\mathsf{CT}}(t) = L(t) \cdot T_{\mathsf{leaves}}(t) + [1 - L(t)] \cdot T_{\mathsf{Stems}}(t) \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (7.1)$$

where $L(t)$ and $[1 - L(t)]$ describe the dry matter proportion of leaves and stems, respectively, in the yield of the tanniferous species during plant development. $T_{\mathsf{leaves}}(t)$ and $T_{\mathsf{Stems}}(t)$ represent the dynamic of the tannin concentrations in leaves and stems over time.

With regard to the tannin concentration of the harvested material of a *mixed* plant community of *one* tanniferous and *one* non-tanniferous plant species, $T_{\mathsf{Mix}}(t)$, competition and changes in the dry matter proportions between tanniferous (p_{CT}) and non-tanniferous plant species ($p_{\overline{\mathsf{CT}}}$) can be integrated into the model as follows:

$$T_{\text{Mix}}(t) = p_{\text{CT}}(t) \cdot T_{\text{CT}}(t) + \overbrace{p_{\overline{\text{CT}}}(t) \cdot \overbrace{T_{\overline{\text{CT}}}(t)}^{=0}}^{=0} \quad \ldots \quad (7.2)$$

$$= p_{\text{CT}}(t) \cdot T_{\text{CT}}(t) \quad \ldots \quad (7.3)$$

$$= p_{\text{CT}}(t) \cdot L(t) \cdot T_{\text{leaves}}(t) + [1 - L(t)] \cdot T_{\text{Stems}}(t)\} \quad \ldots \quad (7.4)$$

More generally, for $1 \ldots n$ tanniferous plant species in competition with each other and with non-tanniferous plants, the tannin concentration of the harvest can be written as:

$$T_{\text{Mix}}(t) = \sum_{1}^{n} p_{\text{CT},i}(t) \cdot T_{\text{CT},i}(t) \quad \ldots \quad (7.5)$$

$$= \sum_{1}^{n} p_{\text{CT},i}(t) \cdot \{L_i(t) \cdot T_{\text{leaves},i}(t) + [1 - L_i(t)] \cdot T_{\text{Stems},i}(t)\} \quad \ldots \quad (7.6)$$

where $p_{\text{CT},i}(t)$ represents the dry matter proportion of the tanniferous plant species i to the total dry matter harvest of the community as a function of time t. $L_i(t)$ stands for the dynamic of the relative dry matter contribution of leaves of the i^{th} tanniferous species to its own dry matter yield. $T_{\text{leaves},i}(t)$ and $T_{\text{stems},i}(t)$ describe the tannin concentration in leaves or stems of the i^{th} tanniferous plant species, respectively, as shown in Chapter 3 (Eqn. 3.3 on page 58 of this thesis).

Note that at any particular point in time, t', the sum $\sum_1^n p_{\text{CT},i}(t = t')$ equals one *only in case* that there are no non-tanniferous species in the community. Otherwise, $\sum_1^n p_{\text{CT},i}(t = t')$ equals the sum of the proportions of all tanniferous plant species to the dry matter yield of the community.

The outlined modelling approach meets all the requirements formulated in previous sections and chapters: It is bare of any assumptions concerning the functionality of the modelled compounds (in this case, condensed tannins); it allows an inclusion of knowledge of biosynthestic regulation mechanisms (any regulation mechanism or dependence of external factors that can be formulated mathematically can be included into the model)[3]; it acknowleges dilution not only as a nuisance parameter[4] but as an

[3]For example: it has been shown that in the presence of an insectan elicitor (j=1, otherwise j=0) the tannin concentration in the leaves of the i^{th} species of a mixed stand increases by 29 %. This physiological plant response could be included into the model by replacing $T_{\text{leaves},i}$ by $T_{\text{leaves},i,j}$ = $T_{\text{leaves},i} + j \cdot 0.29 \cdot T_{\text{leaves},i}$.)

[4]See the discussion of Chapter 3.

important determinant of secondary metabolite concentrations and its estimates are explicit and quantitative (i.e. g CT kg^{-1} DM d^{-1}). In the next section the relative importance of the different components of the model shall be analyzed and discussed.

What are the most important determinants of the tannin concentration of the harvested plant material? – A model interpretation.

The results presented in Chapter 3 and 4 led to the model in equation 7.6 on the preceding page which allows an investigation of the relative importance of different potentially influential parameters. The model acknowledges: (i) Competition between plant species with different tannin concentrations in a community. (ii) Shifts in the leaf / stem ratio during plant development. (iii) Changing tannin concentrations within leaves and stems of the tanniferous plant species during their development. The following conclusions can be drawn:

1. **Interspecific competition and growth dynamics:**

   (a) Only tanniferous species with relatively high abundances ($p_{CT,i}$) and ...

   (b) *simultaneous* high[5] tannin concentrations ($T_{CT,i}$) can contribute to the tannin concentration of the dry matter yield of the mixture (T_{Mix}).

   Species that are either rare or have very low tannin concentrations – or species that have both high dry matter proportions and high tannin concentrations but not simultaneously – cannot be influential on the tannin concentration of the total harvest of the mixture![6]

2. **Biomass allocation and morphology:** In Chapter 3 it has been shown that changing proportions of leaves and stems can be influential for the determination of the tannin concentration of the harvestable aboveground biomass of the tanniferous species. Considering equation 7.6 on the facing page, it can be seen that for the prediction of the tannin concentration of a plant community, the leaf mass fraction of the i^{th} species, $L_i(t)$, can only be important if...

[5] A *high* tannin concentration in this context means high in comparison to the other plant species of the plant community. It is important to note that the tannin concentration ($T_{CT,i}$) cannot exceed the weight of the tissue in which it is found. In case that the tannin concentration is expressed as a percentage of the tissue's dry weight, $T_{CT,i}$ has a lower boundary of zero and an upper boundary of one.

[6] If either $p_{CT,i}$ or $T_{CT,i}$ are small, $p_{CT,i} \cdot T_{CT,i}$, and thus, the contribution to the tannin concentration of the harvest of the mixture becomes small.

(a) the species is abundant in the community (at least sometimes) and...

(b) *at the same time* has high tannin concentrations in either its leaves or stems (but not in both organs concurrently; the difference between $T_{\text{leaves},i}$ and $T_{\text{stems},i}$ needs to be large; see Chapter 3) and ...

(c) the leaf / stem ratio changes strongly over time during the period the species is abundant.

3. **Environmental factors and plant physiology:** Chapter 2 demonstrated that environmental factors like the fertility of the soil or the presence of plant 'enemies' can influence the tannin concentrations of plant tissues. With respect to the tannin concentration of the harvest of a plant community, such parameters need only be considered if...

   (a) they *strongly* affect the tannin concentration of...

   (b) a *large fraction* of tanniferous plant species...

   (c) that are *abundant* in the community (at least sometimes) and,...

   (d) simultaneously, have *high* tannin concentrations relative to the other species in the community (at least sometimes).

   In all other situations, factors that influence tannin concentrations of particular plant tissues of tanniferous plant species in a community are of little relevance for the determination of the tannin concentrations of the dry matter yield of the entire community.

Based on the above analysis, I suggest that the importance of the various potentially influential parameters is hierarchical and conditional: If competition within a sward between plants with strongly differing tannin concentrations plays a major role, the leaf / stem ratio of certain species and changes of the tannin concentrations within the leaves and stems of certain species cannot. On the other hand, if competition is relatively unimportant (e.g. in an almost pure stand of tanniferous plants) the leaf / stem ratio can be important; i.e. when the difference in tannin concentrations between leaves and stems is large and the leaf / stem ratio varies strongly during the observation period. With regard to the tannin concentration of the total dry matter yield of the mixture, changes of the tannin concentrations in particular tissues are of limited importance when either competition or the leaf / stem ratio play an important role.

In short, only changes in tissues that make up a substantial fraction of both, the dry matter yield and the total amount of tannin in the harvest can be important. Therefore,

attempts that aim at a modification of the tannin concentration of the tanniferous plant species' tissues by breeding or by means of genetic engeneering (e.g. Marles et al., 2003; Aerts et al., 1999)[7] should be evaluated very carefully; with regard to the tannin concentration of the field grown harvest, any loss of competitive ability of the tanniferous forage might more than outweigh the gain in tannin concentration of the leaves. Apart from such technical considerations and to the extent that the goal is to apply tanniferous forages against gastrointestinal parasites in *organic* farming, it seems more than questionable wether genetic engeneering is the best approach to increase the tannin concentration of the harvested material.

7.2 Agronomic potential of tanniferous forage plants

This thesis confirmed the potential of tanniferous forage plants for the practical application against gastrointestinal parasites in sheep. Especially *Onobrychis viciifolia* and *Lotus corniculatus* appear to be promising candidate plants: They are commercially available, have moderate tannin concentrations and are highly productive (Chapter 4: 10–13 t DM ha^{-1} y^{-1} when sown as pure stands). Both species are highly palatable despite their elevated concentrations of condensed tannins (see Chapter 4) and effective against some of the most important sheep parasites (Chapter 5). For *Onobrychis viciifolia* it was demonstrated that the antiparasitic effect was maintained even when the forage was dried or ensiled (see Chapter 6).

As a disadvantage, stands of *Onobrychis viciifolia* and *Lotus corniculatus* were subject to pronounced fluctuations of the dry matter contribution of these species to total yield due to either invasion of unsown species (when sown as pure stands) or due to the temporal dominance of the partner-grass of the mixture (e.g. *Festuca pratensis*), leading to highly variable tannin concentrations in the dry matter yield of the experimental fields during the season (Chapters 4 and 5). The fact that there was some variability in the competitive ability of the various cultivars indicates some potential for improvement of the competitive ability of the tanniferous species by breeding. It should be noted,

[7] In their review of the molecular regulation of proanthocyanidin biosynthesis, Marles et al. (2003) concluded that *the manipulation of proanthocyanidins in valuable crops is nearly at hand.* Aerts et al. (1999) wrote that *recent investigations may ultimately enable the expression by genetic engineering of increased levels of PA in the leaves of agriculturally important forage plants such as white clover and perennial rye grass.*

however, that in the case of *Onobrychis* fluctuations of the dry matter proportion of the tanniferous plant species to total yield were related to the growth dynamic of this species rather than to its competitive ability (see Fig. 4.2 on page 89 and the pictures on page 185 in Appendix B); it is known that *Onobrychis viciifolia* is very productive in spring and early summer but not so much in autumn (Borreani et al., 2003), leading to dominance of unsown species or mixing partners in the second half of the vegetation period. For the use against gastrointestinal parasistes and to the extent that it is desirable to have forage with high tannin concentrations, it might be advisable to use only the spring and early summer harvests as anthelmintic forage while the autumn harvest can be used for other purposes. In this context, it is relevant that we could demonstrate antiparasitic effects of hay and silage (Chapter 6), meaning that the anthelmintic forage can be conserved, potentially traded with other farmers and administered when this is appropriate with regard to the ruminant's health.

As shown in Chapters 3, 4 and 5, *Cichorium intybus* has only very low concentrations of condensed tannins which are unlikely to be responsible for the sometimes reported antiparasitic effect of this species (e.g. Marley et al., 2003) which can perhaps be attributed to its content of sesquiterpene-lactones (see the discussion of Chapter 5; Hoste et al., 2006; Molan et al., 2003). Apart from its potential as anthelmintic forage, *Cichorium intybus* is known for its relatively high concentrations of minerals and has been reported to increase the milk production of dairy cows (Jung et al., 1996). In our experiments, *Cichorium intybus* produced a highly palatable dry matter yield between 10 and 13 t DM ha^{-1} y^{-1} (Chapter 4). Especially the cultivar 'grasslands Puna' has a high potential as a valuable forage plant. In comparison to the other tested cultivars[8], *Cichorium intybus* 'grasslands Puna' excelled by producing a high yield with a minimum production of stems. All tested cultivars of *Cichorium intybus* proved amazingly resistant to weed invasion; even when sown in mixture with *Festuca pratensis*, *Cichorium* grew almost in monoculture. Based on visual assessments of the swards during the experiment and on the relatively large patches of open soil which stayed often uncovered for several weeks, I seriously doubt that the competitve ability of *Cichorium intybus* can solely be attributed to its large leaves and a competitive advantage for light. Allelopathic effects seem likely, though there was no conclusive evidence to support this suspicion. Because of its high water content, *Cichorium* should not be administered fresh to young

[8]The performance of *Cichorium intybus* cv. INIA lacerta, cv. Forage Feast, cv. grasslands Puna and cv. INIA la Niña have been evaluated in this thesis. This cultivar INIA la Niña was eliminated early, after a poor performance (low yield, susceptible to fungi) in a preliminary trial in 2003 and therefore is not mentioned elsewhere in this thesis.

animals (see the discussion of Chapter 5).

The relatively high concentrations of condensed tannins previously reported for *Lotus pedunculatus* (e.g. Min et al., 2003; Aerts et al., 1999) were confirmed in our experiments. However, this species proved unsuitable for cultivation under 'normal' agronomic conditions (see Chapters 4 and 5). Maybe more than for its agronomic potential, the species is interesting for its tannins and might prove useful to clarify the relationship between the chemical structure of condensed tannins and their effect on ruminants; tannins of *Lotus pedunculatus* have been reported to have detrimental effects on ruminants whereas the tannins of the relatively closely related *Lotus corniculatus* usually produce positive outcomes (Mueller-Harvey, 2006; Min et al., 2003; Aerts et al., 1999).

7.3 Outlook

Future research should focus on the the following two main areas:

1. A clarification of the relationship between the chemical structures of condensed tannins and their mode of action with regard to parasites and ruminants.

   (a) An in-vivo dose-response curve obtained with purified forage plant-derived condensed tannins against gastrointestinal parasites is highly desirable. The extraction and purification of an appropriate amount of condensed tannins will most likely require a cooperation with an experienced industrial laboratory. Once such a dose-response curve has been established, one could continue to compare the efficacy of tannins from various plant species and concurrently analyse their chemical composition.

   (b) With respect to the ruminant, a particularly promising approach seems to me an investigation of the binding strengthof different tannins to RUBISCO which is the quantitatively most important plant protein; interesting approaches include the radial diffusion method with RUBISCO instead of BSA (Jackson et al., 1996) and isothermal titration calimetry (Mueller-Harvey, 2006). This should be combined with an analysis of the chemical structures and the nutritive effects of the various tannins on ruminants.

   (c) If, as Marles et al. (2003) announced, it will soon be possible to quantitatively and structurally modify condensed tannins in forage plants, that could

certainly help to clarify the relationship between the chemical structure of condensed tannins and their mode of action.

2. The development and scientific assessment of possible tanniferous forage-base control strategies against gastrointestinal parasites on farms. This should be combined with practice-ortiented research to improve these strategies and a concurrent evaluation of the farmers acceptance of the procedure.

   (a) Often it was found that feeding of tanniferous forage plants reduced the faecal parasite egg output, implying a long-term benefit of a decreasing pasture contamination. Research should focus on how administration of tanniferous forages and pasture rotation can be combined to minimize the risk of reinfections.

   (b) Especially in *Onobrychis viciifolia*, which I consider the most promising of the tested forages, tannin concentrations of the field-grown harvest was not constant during the season (due to competion and differences in growth rates between *Onobrychis viciifolia* and competing non-tanniferous plant species). Research should focus on wether it is possible to improve its competitive ability and its productivity in autumn, to enhance its leaf / stem ratio and perhaps to increase its tannin concentration. In order to achieve higher and more constant contributions of *Onobrychis viciifolia* to the total dry matter yield, adaptations of the species mixtures (other mixing partners; see the discussion in Chapter 4) and management practices (N-input, cutting frequences) should also be considered.

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Appendix A

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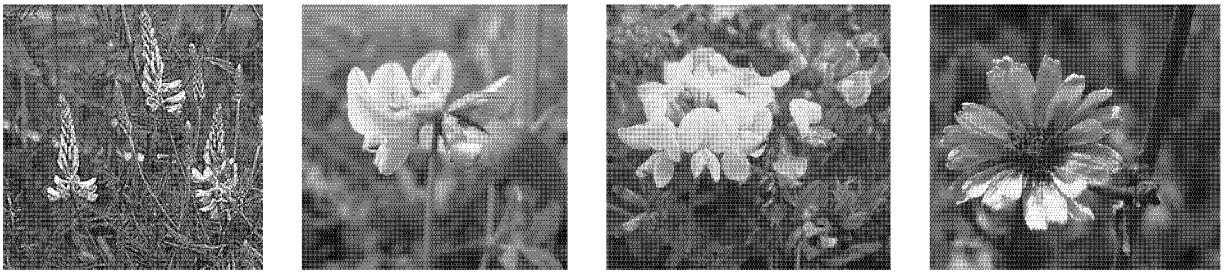
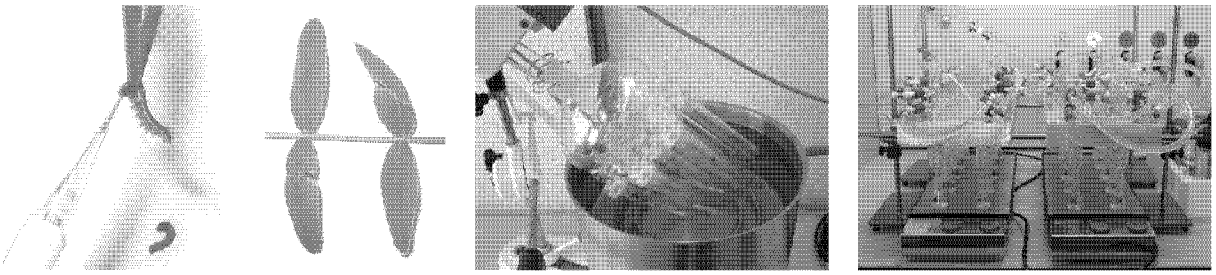
Appendix B

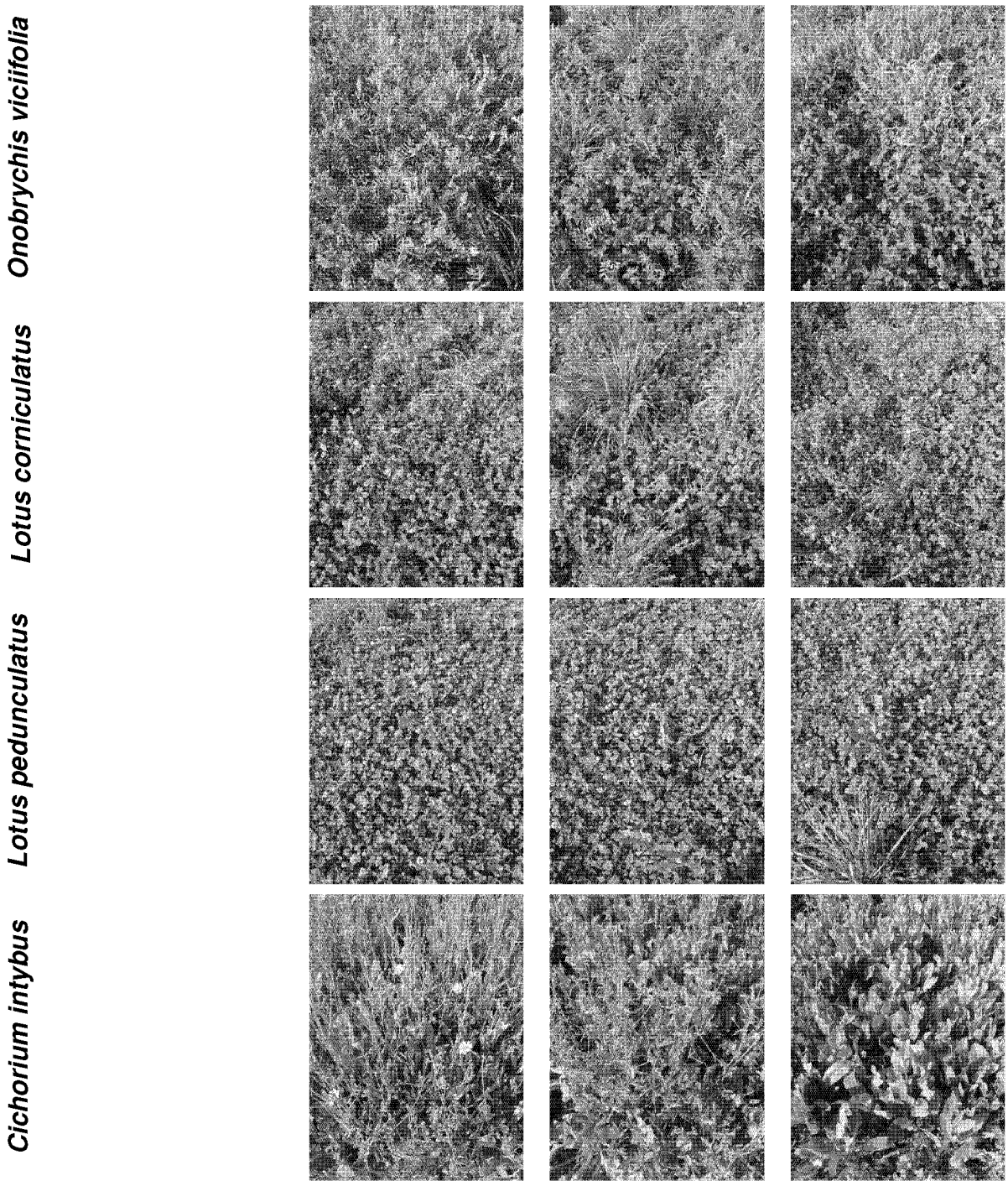
Photographs



From left to right: Flowers of Onobrychis viciifolia*,* Lotus corniculatus*,* Lotus pedunculatus, Cichorium intybus*.*

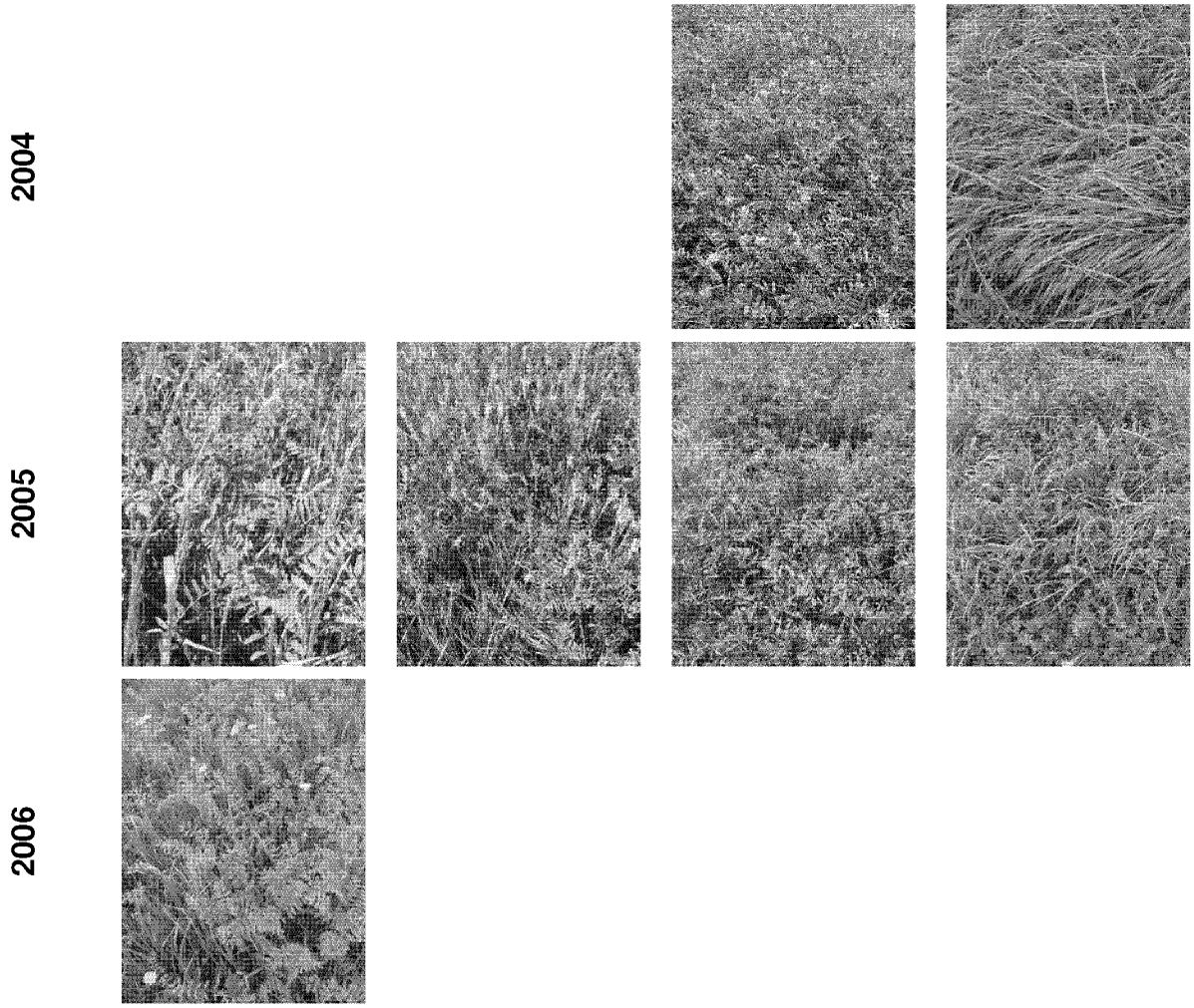


From left to right: Collecting caterpillar's spit from Spodoptera littoralis*. The wounds on* Onobrychis viccifolia *at the end of the experiment (Chapter 2). A rotoevaporater-spider with 20 tubes. Samples were heated under reflux.*



Purely sown stands of Onobrychis viciifolia *cv. Visnovsky, commercial seed, Alvaschein;* Lotus corniculatus *cv. Lotar, Odenwälder, Oberhaunstädter;* Lotus pedunculatus *cv. sunrise, maku, barsilvi;* Cichorium intybus *cv. INIA Lacerta, grasslands Puna, Forage Feast. Pictures have been taken in the 30th July 2005 (second year of the experiment).*

Left: Germination on the experimental field. Center: Harvest of the experimental field (the yield is directly weighed on the machine). Right: Collecting botanical samples on the field.



Mixed stand of Onobrychis viciifolia *cv. Visnovsky and* Festuca pratensis *cv. Preval. From the upper left to the lower right corner, pictures have been taken 3rd August 2004, 12th October 2004, 1st June 2005, 30th July 2005, 26th August 2005, 10th October 2005, 8th May 2006. Note the seasonal change in the proportions of tanniferous and non-tanniferous plants.*

Appendix C

Danksagungen

Ganz herzlichen Dank an alle[1], die direkt oder indirekt zum Erfolg meiner Dissertation beigetragen haben! Besonders bedanke ich mich bei **Prof. Nikolaus Amrhein**, meinem Doktorvater, und bei **Prof. Peter Edwards** für das Vertrauen in mein selbständiges Arbeiten und die kritische Beurteilung der Manuskripte. Mein grösster Dank gilt aber **PD Dr. Andreas Lüscher** und **Dr. Daniel Suter**, die die Doktorarbeit ermöglicht und vor Ort begleitet haben. Danke, dass Ihr geholfen habt, das Tannin-Projekt ins Leben zu rufen; das war und ist eine interessante Sache! Vielen Dank für Eure Unterstützung und Euer Vertrauen bei der Planung und der Durchführung der Experimente, die angenehme Atmosphäre am Reckenholz und für die Finanzierung diverser Hilfskräfte, ohne die eine Dissertation in diesem Umfang nicht möglich gewesen wäre. Für die Finanzierung bedanke ich mich ausserdem recht herzlich beim Bundesamt für Landwirtschaft in Bern.

Ein grosses Dankeschön geht an **Prof. Thomas Boller** (Universität Basel), **Prof. Georg Felix** (Universität Tübingen), **PD Dr. Peter Salzer** (ehem. Universität Basel), und **Prof. Ted Turlings** (Université de Neuchâtel) für die äusserst grosszügige Versorgung mit Elizitoren. Das war spitze! Danke an **Prof. Tom Barry** und **Dr. Ramirez-Restrepo** von der Massey University in Neuseeland für die Zurverfügungstellung von voranalysierten Tanninproben. Diese äusserst wichtige Starthilfe hat mir entscheidend dabei geholfen, die Tannin-Analyse am Reckenholz zu etablieren und deren Resultaten zu vertrauen.

Besten Dank an meine Mit-Doktoranden der beiden anderen Module des Tannin-Projektes: **Felix Heckendorn** (Parasitologie) und **Anna Scharenberg** (Wiederkäuerernährung) – die Zusammenarbeit mit Euch hat Spass gemacht. Speziell hervorheben möchte ich die intensive, fordernde und fruchtbare (siehe die beiden letzen Kapitel dieser Dissertation) Zusammenarbeit mit Felix in zwei Experimenten am FiBL und beim Schreiben der Manuskripte: Wir waren uns nicht immer einig, konnten uns aber immer einigen! Ich bin stolz und freue mich am Resultat dieser Zusammenarbeit. Danke auch an **Dr. Frigga Dohme**, **Dr. Andreas Gutzwiller**, **Dr. Hubertus Hertzberg**, **Dr. Veronika Maurer**, **Aurélia Perroud** und **Prof. Michael Kreuzer**. Ich empfand die Interdisziplinarität des *Tannin-Projektes* zuweilen als anstrengend aber stets auch als interessante Herausforderung. Es ist gut, hin und wieder gezwungen zu sein, über den eigenen Tellerrand zu sehen ...

[1] Die Verwendung oder vielmehr der Verzicht auf die Verwendung akademischer Titel in der Danksagung unterliegt einer gewissen Willkür und meiner persönlichen Einschätzung meiner Beziehung zur betreffenden Person. Ich bitte dafür um Verständnis.

Vielen Dank an **Hans-Ueli Briner**, **Erich Rosenberg** sowie an deren diverse Praktikanten und Praktikantinnen für die Unterstützung bei den Feldarbeiten. Danke an **Dr. Hans Jörg Bachmann** und **Dr. Hans Stünzi** für den Arbeitsplatz im D-Stock, vor allem aber an **Hans-Ruedi Bosshard** und an das ganze D-Stock Chemie-Team am Reckenholz, ihr wart super! Merci an **Dr. Franco Widmer** und **Dr. Rolland Kölliker** für die Zentrifuge und die Sterilbank sowie an **Philipp Streckeisen**, für die Betreuung der Gewächshäuser. Danke den vielen fleissigen Praktikanten und Zivildienstlern, die bei mir auf dem Feld, an der Mühle und im Labor mitgeholfen haben. Es sind dies: **Kirsti Määtänen**, **Stéphane Voléry**, **Fabian Neve**, **Matthias Müller**, **Philipp Kadelbach**, **Katja Lüscher**, **Michael Huber**, **Matthias Löpfe** (der schamanistische Lötfisch), und **Matthias Erb**. Danke auch an die Baracken-Mitbewohner und späteren F-Stöckler des Reckenholzes: **Dorothea Kampmann**, **Markus Hohl**, **Markus Peter**, **Daniel Nyfeler**. Es war spannend, hitzig und lustig mit Euch. Mit Euch immer wieder ...

Danke an diverse andere Mitarbeiter des Reckenholzes. Spontan kommen mir **Olivier Huguenin**, **Bruno Studer**, **Martin Hartmann**, **Daniel Berner**, **Serge Buholzer**, **Irene Weihermann**, **Willy Kessler**, **Maya Bossard**, **Daniel Baumgartner**, **Armin Hediger**, **Françoise Roth**, **Philippe Jeanneret**, **Regula Kistler**, **Jens Leifeld**, **Urs Nyffeler**, **Cornel Stutz**, **Raffael Gago**, **Matthias Suter**, **Mario Waldburger** und **Priska Gassmann** in den Sinn. Vielen Dank an Euch und an alle anderen Mitarbeiter des Reckenholzes, die hier versehentlich nicht erwähnt sind.

Günter Hoch danke ich für die Analyse der Kohlenhydrate. Mindestens ebensosehr verbunden fühle ich mich ihm, sowie **Gabriella Schaer**, **Eva Spehn** und allen anderen Mitarbeitern des Botanischen Institutes in Basel, die ich noch kenne, für den jeweils herzlichen Empfang an der Schönbeinstrasse 6. Schön, dass man sich bei Euch auch nach vier Jahren Abwesenheit immer noch ein bisschen zuhause fühlt.

Obwohl eigentlich nicht direkt an meiner Dissertation beteiligt, bedanke ich mich ganz besonders bei **Prof. Christian Körner** von der Universität Basel. Mit seinen packenden Vorlesungen, seinen inspirierenden Ideen und vor allem mit seiner eigenen Begeisterung für die Forschung hat er das Feuer für Pflanzenwissenschaften auch in mir entfacht. Danke auch für die Ausbildung während des Studiums und der Diplomarbeit. Die bei der Diplomarbeit gesammelte Erfahrung beim Schreiben eines Artikels hat mir enorm geholfen, das Licht am Ende des Tunnels auch während der Dissertation früh zu erahnen und es in der Folge nicht mehr aus den Augen zu verlieren.

Nicht zu vergessen sind die vielen **fleissigen Programmierer**, die unermüdlich an tollen freeware Programmen wie LaTeX, R, oder dem Gimp rumbasteln. Nicht auszumahlen mit welchen Mühsalen man sich sonst abgeben müsste. Dank euch war es eine richtige Freude, das Diss-Büchlein zu erstellen.

Ein Hoch auf meine Weggefährten **Sebastian Leuzinger McClintock** und **Thomas Fabbro** und auf die Weggefährtin **Stephanie von Felten**, mit denen ich bereits in Basel Biologie und anschliessend in Neuchâtel Statistik studiert habe. Das war zuweilen wirklich hart! Danke für Eure mathematische und vor allem auch moralische Unterstützung. Ich weiss nicht, ob ich das ohne Euch durchgezogen hätte. Also: Hipp, hipp, hurra! Auf Euch – auf mich – AUF UNS!

Ein Riesendank gebührt meinem guten Freund **Sergey Studer**; auf Dich ist Verlass wenn's brennt! Herzlichen Dank auch an all meine Kollegen, für Ablenkung und psychologische Betreuung in schlechten Zeiten und für beste Unterhaltung in guten Zeiten: **Raymond Petitjean**, **Dani Haus**, **Mathis Stoeckle**, **Jan Stucki**, **Sabine Nurnus**, **Anne Scheibler**, **Martin Dürrenberger und Linda Zimmermann**, **Röbi und Pia Suter**, **Alana McClintock**, **Barbara Brunner**, **Thomas Sattler**, **Oliver Gardi**, **Patrick Gasser**.

Besonderer Dank gilt meiner Frau **Monika**, die durch ihre tatkräftige und moralische Unterstützung während der ganzen Doktorarbeit, vor allem aber durch Ihr Verständnis und ihre Liebe einen unverzichtbaren Beitrag an das Gelingen dieser Doktorarbeit geleistet hat. Es ist gut zu wissen, dass unsere Liebe auch diese vier Jahre Gependel zwischen Basel, Zürich und Neuchâtel ausgehalten hat!

Schliesslich bedanke ich mich bei unseren beiden Familien: In erster Linie bei meinen Eltern, **Madeleine und Werner Häring-Hauptlin**, die mir den Auslandaufenthalt in Australien und das Biologie Studium ermöglicht haben, aber auch bei **Anita Häring** und **Markus Portmann**, bei **Reto Häring und Silke Wendt**, bei **Maria und Urs Flury** und bei **Beatrice Flury und Tommaso Bruno** sowie ihren beiden Söhnen und meinen Neffen **Leandro** und **Ruben** – Ihr alle habt meine Disserattion mit Interesse unterstützend verfolgt und mir in all den Jahren ein stabiles familiäres Umfeld geboten. Vielen Dank!

Appendix D

Curriculum vitae

DIETER ADRIAN HÄRING

Gotthardstrasse 128
4054 Basel
Switzerland

Citizen of Arisdorf, BL
Born 15^{th} of September 1976
Married to Monika Christina Häring-Flury

Education

1993 – 1996	Gymnasium, *Liestal*	**Matur Typus C**
04/97 – 10/97	Stay in Australia, *Sydney*	**Cambridge Certificate in Advanced English**
1997 – 2002	Studies in Biology, *University of Basel*	**Diplom in Biology 1***
2004 – 2006	Studies in Statistics, *University of Neuchâtel*	**Master Degree in Statistics****
2003 – 2007	PhD in Biology, *ETH Zurich*	

Own publications

1. **HÄRING D.A.**, KÖRNER CH. (2004). CO_2 enrichment reduces the relative contribution of latex and latex-related hydrocarbons to biomass in Euphorbia lathyris. *Plant, Cell & Environment*, 27 (2): pp. 209-217.
2. **HÄRING, D.A.**, SUTER, D., AMRHEIN, N., LÜSCHER, A. (2007). Biomass allocation is an important determinant of the tannin concentration in growing plants. *Annals of Botany*, 99 (1): pp. 111-120.
3. **HÄRING, D.A.**, SCHARENBERG, A., HECKENDORN, F., DOHME, F., LÜSCHER, A., MAURER, V., SUTER, D., HERTZBERG, H. Tanniferous forage plants: agronomic performance, palatability and efficacy against parasitic nematodes in sheep. *Renewable Agriculture and Food Systems*, invited article, submitted.
4. **HÄRING, D.A.**, HUBER, M., SUTER, D., EDWARDS, P.J., LÜSCHER, A. Elicitor induced tannin production at four levels of nutrient availability in *Onobrychis viciifolia*. In Progress.
5. HECKENDORN, F., **HÄRING, D.A.**, MAURER, V., ZINNSSTAG, J., LANGHANS, W., HERTZBERG, H. (2006). Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs. *Veterinary Parasitology*, 142: pp. 293-300.

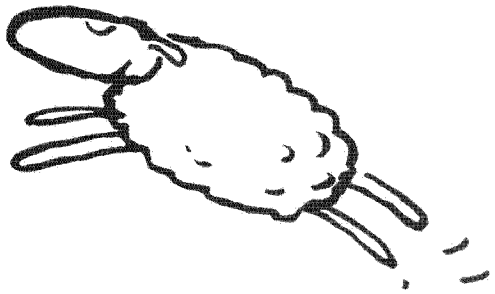
*Diploma thesis: Biomasseproduktion, Entwicklungsdynamik und Sekundärstoffwechsel latexführender Pflanzen unter erhötem CO_2 bei unterschiedlichem Nährstoffangebot, supervised by Prof. Christian Körner, Botanical Institute, University of Basel.

**Master thesis: A Project in Linear Regression: Elicitor induced tannin synthesis at four levels of nutrient availability in *Onobrychis viciifolia*, supervised by Dr. Valentin Rousson, Department of Biostatistics, University Zurich.

6. HECKENDORN, F., **HÄRING, D.A.**, MAURER, V., SENN, M., HERTZBERG, H. (2007). Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemonchus contortus* and *Cooperia curticei*. *Veterinary Parasitology*, 146: 123–134.

Most important contributions to conference proceedings

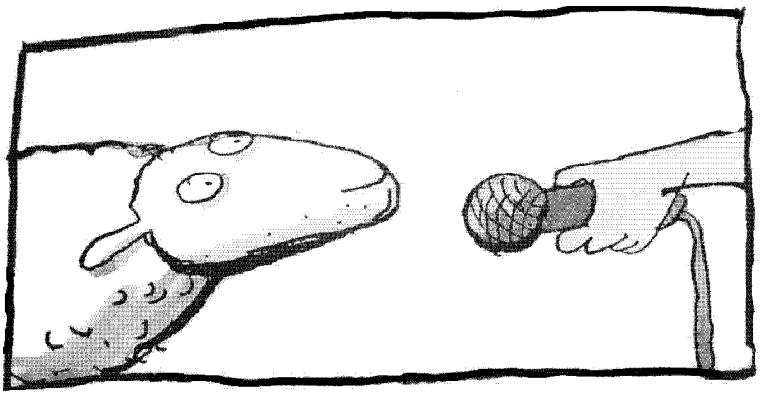
1. **HÄRING, D.A.**, SUTER, D., AMRHEIN, N. AND LÜSCHER, A. (2005) Concentrations of condensed tannins in forage plants as a function of their developmental stage. In: Integrating efficient grassland farming and biodiversity. Proceedings of the 13th International Occasional Symposium of the European Grassland Federation (EGF), 29. – 31.8.2005, Tartu, Estonia. Grassland Science in Europe 10: pp. 431-435.
2. **HÄRING, D.A.**, HECKENDORN, F., SCHARENBERG, A., AMRHEIN, N., DOHME, F., KREUZER, M., LANGHANS, W., LÜSCHER, A., MAURER, V., SUTER, D., HERTZBERG, H. (2005) Tanniniferous plants as a control agent against gastro-intestinal nematodes in ruminants. In: Sward dynamics, N-flows and forage utilization in legume-based systems. Proceedings of the COST 852 workshop, 10. – 12.11.2005, Grado, Italy: pp. 263-267.
3. LÜSCHER, A., **HÄRING, D.A.**, HECKENDORN, F., SCHARENBERG, A., DOHME, F., MAURER, V., HERTZBERG, H. (2005) Use of tanniferous plants against gastro-intestinal nematodes in ruminants: In Researching sustainable systems. Proceedings of the 15th IFOAM Organic World Congress, 21 – 23.09.2005, Adelaide, South Australia: pp. 272-276.



Appendix E

Artwork

Artwork on page iii, 190, 193 and on this page shows *'Selma'* and has been done by Jutta Bauer. Her booklet *'Selma'* is highly recommended to the interested reader[i]. For Artwork on page 32 and 187 the author of this thesis is to blame.



[i] Jutta Bauer (2000) Selma. Lappan Verlag GmbH

Appendix F

List of Figures and Tables

List of Figures

List of Tables