Grain filling, starch degradation and feeding value of maize for ruminants

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Thesis

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Abstract

Maize (*Zea mays* L.) is a major component in the ration of dairy cows in many parts of the world. The currently increasing economic importance of maize has highlighted the need to determine its nutritional value, and to assess the factors influencing its nutritive value. Genotypic make-up (especially differences in starch and endosperm), growing conditions, maturity stage at harvest, and post handling processes, like oven-drying, can influence nutritive value of maize kernels. Similarly, ensiling temperature and duration can affect feeding value of maize silage. This thesis is divided into three parts; the first aim was to characterize the dry matter (starch) accumulation of maize different genotypes in different environments under controlled (glasshouse) and on different locations (sand and clay) in field conditions. Maize genotypes used were different in starch structure and composition, and in type of endosperm. Starch structure refers to amylose and amylopectin; and composition refers to their proportions, whereas type of endosperm defines levels of vitreousness. The vitreousness is the ratio of vitreous (hard) to floury (soft) endosperm. Six maize genotypes, differing in amylose content and vitreousness, were grown under three contrasting day/night temperature regimes during grain filling and harvested at different maturity stages from two greenhouse experiments. Similar investigations were carried on another set of genotypes grown on sandy and clay soils and with different sowing times under field conditions. Water contents and dry matter (starch) accumulation were significantly influenced by growth temperature, genotype, soil type and sowing time ($P<0.0001$). The second aim of thesis was to establish a relationship between rumen *in vitro* starch degradation (feeding value) of maize kernels and different factors, like genotype, growth temperature during grain filling, and maturity stage. Oven-dried kernels of six maize genotypes, from the two greenhouse experiments mentioned before were investigated. Starch content was measured using an enzymatic method and the gas production technique was used to assess starch degradation in rumen fluid of dairy cows. The extent of starch degradation at different incubation times was calculated from measured gas production data (6, 12 and 20 h, respectively) and a published equation. At each maturity stage, whole kernel and starch degradation in rumen fluid

depended on the genotype (P<0.0001), growing conditions (P<0.0001), starch content (P<0.0001) and starch amount (P<0.0001) in the kernels. The same but fresh (not oven-dried) maize kernel samples were investigated using gas production technique to determine the impact of oven-drying on rumen *in vitro* starch degradation of maize kernels. Oven-drying significantly (P<0.0001) influenced the rumen *in vitro* starch degradation in maize kernels various incubation times, with more starch being degraded in the fresh than in the oven-dried maize kernels, although the differences were small. There was a consistent and highly significant (P<0.009 to 0.0002) interaction between oven-drying and genotype, with the high-amylose genotype showing larger effects of oven-drying than the other genotypes. The third aim of thesis was to investigate effect of ensiling temperature and duration on feeding value of maize silage. Samples of maize whole plants (dry matter 33%) were collected from the medium vitreous endosperm cultivar, grown in different seasons on sandy soils. Maize plants were chopped and ensiled in mini silos at three different temperatures. Samples from the silos were taken after 0 (not ensiled, i.e. control), 4, 8 and 16 weeks of ensiling. The gas production technique was used to evaluate the influence of the ensiling temperature and duration of ensiling on the degradation of the fresh ground silage samples in rumen fluid. The final pH of the silages and the gas production was significantly influenced by ensiling temperature in both seasons (P<0.0001). Gas production and pH decreased with an increase in ensiling duration (P<0.0001). The relationship between pH and gas production was quadratic and depended on the ensiling temperature (P<0.002). It was found that ensiling temperature and ensiling duration determine the rate of change and final pH, and play a significant role in feeding value of maize silage. The finding of thesis can be used to determine the exact feeding value of maize kernels and silage, and also can be used as a tool to revise the current feeding evaluation systems i.e. shift from oven-dried to fresh samples.

Keywords; Maize (*Zea mays* L), Genotypes, Grain filling, Growth temperature, Kernels, Gas production, Starch degradation, Oven-drying, Silage, Ensiling temperature, Ensiling duration, Feeding value, Lactating cows

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General introduction

Chapter 1

Maize (*Zea mays* L.) is a plant grown worldwide, although according to FAO Stat, over 80% of maize production occurs in the Americas (50%) and Asia (31%). Over the past 20 years, maize production has shown a steady increase, especially in Asia (FAO Stat, 2013). Maize is an extremely versatile crop. It is a large leafy stalk grain plant which produces cobs that contain the grain (seeds, also called kernels). Maize kernels are used as food and feed or as feedstock for bio-ethanol production as they mainly contain starch. Maize kernels consist of 82% endosperm, 12% germ, 5% bran and 1% tip cap. In terms of composition, maize kernels contain 61% starch, 8% protein, 4% oil and 13% ash and fibre and 14% water. On the basis of dry matter, maize kernels contain 71% starch, 9% protein, 5% oil and 15% ash and fibre.

Whole-crop maize is a major component in the ration of dairy cows in many parts of the world. The increasing economic concern and importance of maize has highlighted the need to determine its nutritional value, and the factors which influence its nutritive value (Garcıa-Rodriguez *et al*., 2005). Whole-plant maize has a relatively high degradability (approximately 72-75% on an organic matter basis), mainly due to its high content of kernels and the high starch content in these kernels. Therefore, to understand the true feeding value of maize in dairy rations, it is essential to have a good understanding of the degradability of maize kernel starch as well as of the other components in the plant.

Degradability of starch in maize kernels is influenced by intrinsic and environmental conditions, such as plant age, growing conditions and post-harvest treatments (Fahey *et al.*, 1993). The rate and extent of rumen starch degradability can be highly variable (Orskov, 1986; Owens *et al*., 1986; Rooney and Pflugfelder, 1986; Theurer, 1986; Nocek and Tamminga, 1991). This variability is associated with diversity in starch structure and composition and also with the diversity in type of endosperm among different maize genotypes. Different maize genotypes produce kernels with varying contents and composition of starch (i.e. amylose or amylopectin). Biochemical modification in kernel starch biosynthesis due to genetic differences is complex (Hageman and Lambert, 1988). Consequently, the genotypes can differ in rumen starch degradation and so in feeding value (Frei, 2000; Troyer,

2000; Duvick, 2005). Therefore, the feeding value of maize for ruminants depends largely on the yield and the starch content of the ears.

Starch provides an important energy source for animal growth and milk production, and has traditionally been considered the most important characteristic of maize silage quality. As starch is relatively inert during the ensiling fermentation process, very little starch is lost in the silage in contrast to, for example, water-soluble carbohydrates. The latter components may be lost through fermentation processes or through seepage during storage. This means that the process of starch formation, whether it is based on on-going photosynthesis or on re-allocation of water soluble carbohydrates from the stem, greatly contributes to the production of storable dry matter.

As starch in maize kernels plays an important role in determining the feeding value of maize for ruminants, understanding starch in terms of structure, composition and characteristics is important for improvement in animal performance.

What is starch?

The word "starch" is derived from the Middle English “sterchen”, meaning to stiffen. "Amylum" is Latin for starch, from the Greek “amylon" which means "not ground at a mill". The present definition of starch is: a carbohydrate consisting of a large number of glucose units joined by glycosidic bonds. This polysaccharide is produced by plants as an energy store.

In photosynthesis, plants use light energy to produce glucose from carbon dioxide and water. The glucose is stored mainly in the form of starch granules, in plastids such as chloroplasts and especially amyloplasts. Toward the end of the growing season, starch accumulates in twigs of trees near the buds. Fruits, seeds, rhizomes, and tubers store starch to prepare for the next growing season.

Starch structure and composition

Starch is composed of two distinct polymers, amylose and amylopectin (Fig. 1). Amylose is linear, has a relatively low molecular weight and is composed of alpha-1,4-linked glucose units. Amylopectin is an alpha-1,4-linked, alpha-1,6-branched (4–6% branching) polymer with a relatively high molecular weight (Jackson, 2003). Normal maize starches are mixtures of amylose (20-30%) and amylopectin (70-80%) and different starch composition generally reflects differences in activities of enzymes involved in starch synthesis. Starch composition is known to vary among and within plant species (Fankhauser *et al*., 1989).

The starch can be described in terms of physio-chemical properties of the amylose and amylopectin, variation in their composition, molecular level interactions and their associations, and the macro level of starch granules (Tester *et al*., 2004).

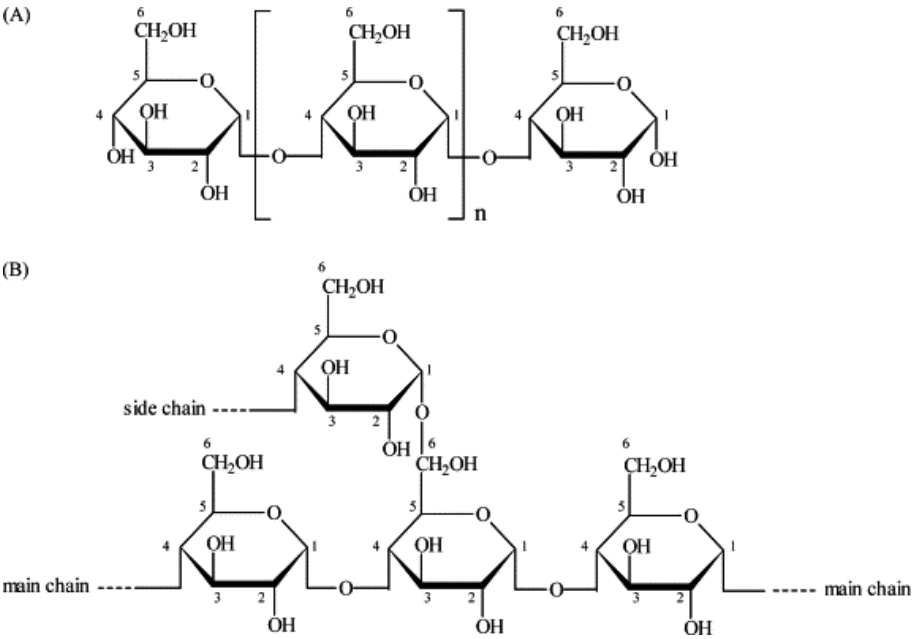

Fig. 1. Structure of the amylose (A) and amylopectin molecule (B).
Source: Herrero-Martínez *et al*. (2004).

Starch biosynthesis

Starch biosynthesis, as shown in Fig. 2, is a complex phenomenon (Tester *et al*., 2004). It starts with alpha-glucan deposition in sucrose (derived from photosynthesis). The sucrose is converted to uridine diphosphate glucose (UDP-glucose) and fructose by sucrose synthase in the cell cytosol. The UDP-glucose is then converted to glucose-1-phosphate (G-1-P) by UDP-glucose pyrophosphorylase in the presence of pyrophosphate (PPi). G-1-P is then itself converted to glucose-6-phosphate (G-6-P) by phosphoglucomutase. The G-6-P is translocated by specific translocators across the amyloplast membrane (the intra-cellular organelle responsible for starch biosynthesis in storage tissues) and is converted to G-1-P by phosphoglucomutase. There is evidence of two possibilities; one is that, in cereals at least, G-1-P may be translocated directly into the amyloplast. Second possibility is that G-1-P is converted to, and translocated as, adenosine diphosphate glucose (ADP-glucose), produced as a consequence of adenosine diphosphate (ADP)-glucose pyrophosphorylase (cytosol based) activity in the presence of adenosine triphosphate (ATP). G-1-P within the amyloplast is (also) converted to ADP-glucose by using ADP-glucose pyrophosphorylase. This ADP-glucose provides glucose residues for amylose and amylopectin biosynthesis.

Starch synthase enzymes (commonly considered to be of two major classes, ‘granule bound’ and ‘soluble’) add glucose units to the non-reducing ends of amylose and amylopectin molecules. Granule bound starch synthase is responsible for the synthesis of amylose, as it can elongate malto-oligosaccharides to form amylose. Soluble starch synthase is considered to be responsible for the synthesis of unit chains of amylopectin (Tester *et al*., 2004).

Maize starch importance in feeding system

As discussed above, starch plays an important role in the feeding quality of maize for dairy cows. However, the current feeding systems (e.g. in Europe: Van Es, 1978; INRA, 1989; AFRC, 1990 and 1993) do not distinct in starch quality based on differences in rate and extent of starch degradability, and so starch quality does not

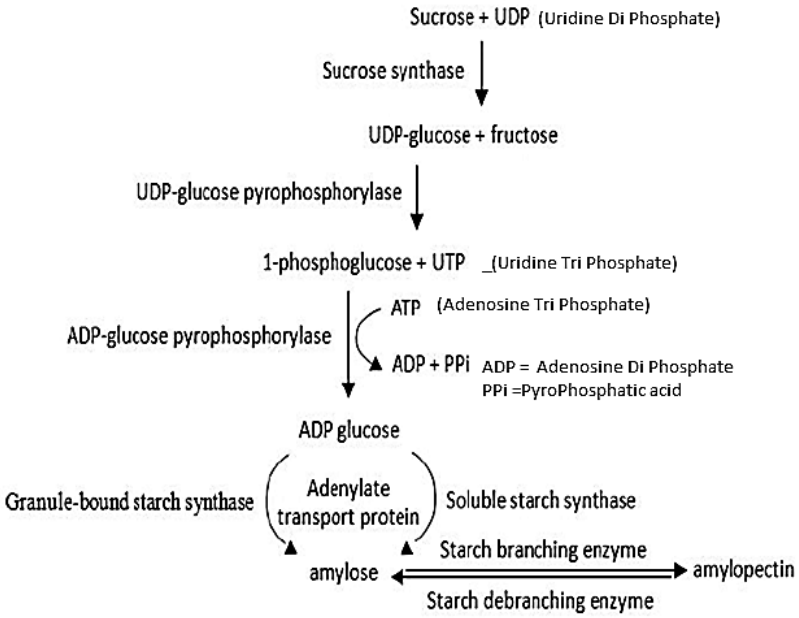

Fig. 2. Starch biosynthesis pathway in maize endosperm.
Source: Yang *et al*. (2011).

influence predicted feeding value. Variation (due to genetic or environmental factors) in this degradation is thus not taken into account. However, literature shows that this variation in the degradation of starch is indeed of interest. Starch, to a large extent depending on its properties, can be broken down in the rumen of ruminants (unsettled starch), or to flow through the rumen and is then broken down in the small intestine (vitreous or rumen escape starch). Depending on the energy needs of the animal (mainly determined by the phase of lactation) the farmer can thus choose a particular type of maize with certain starch properties. For early lactation, when the animals are in a negative energy balance, farmers will opt for resistant starch (vitreous) and later in lactation for less resistant starch (non-vitreous).

Much effort has been focussed on exploring the genotypic factors causing differences in degradability of the cell wall (Buxton and Casler, 1993). However, hardly any research has been done on the quality of starch in the maize kernels. It is not precisely known how the genotypes, different in starch structure and

composition of starch, influence the starch degradation. Maturation effects on quality of starch in those genotypes, and on its degradability still remains to be investigated. In addition, studies (Behall *et al*., 1988; Cone *et al*., 1992) show that starch degradability can also be influenced by their relative chemical composition of the starches of different origin, i.e. its amylose:amylopectin ratio, with amylopectin being better degradable than amylose. However, the degree of degradability was not found to be associated with chemical composition in potato starch. This suggests that starch degradation is influenced not only by chemical composition of the starch but also by physical properties, i.e. gelatination enthalpy (Wolters and Cone, 1992). Kassenbeck (1975) and Cone (1991) showed that a peripheral layer, containing lipids and proteins can also hinder degradation of starch kernels.

Factors affecting starch accumulation in maize kernels, its rate and duration

Genotypes

As explained earlier, starch biosynthesis in maize kernels is an enzyme dependent process which can be influenced by genetic make-up of the maize plant and by growing conditions. In maize, there is a large genetic variation in starch structure and composition (Wilson *et al*., 2004), partly associated with the occurrence of many starch mutants, including waxy and high-amylose types. On the basis of starch structure and composition, maize can be categorized into different genotypes or groups, e.g. dent, flint, waxy, high amylose, vitreous, non-vitreous, etc. These all have their own niche in human or animal nutrition and industrial uses, but the dent type is the one most commonly used as animal feed when only the kernels are fed. In whole-plant maize silages both flint and dent are commonly used with the flint types more abundant at the northern cooler borders of the ecological distribution of the crop, due to their cold tolerance.

Dent corn (*Zea mays indentata*) is often used as livestock feed, in industrial products, or to make processed foods. Dent corn is also frequently referred to as "field" corn. Either white or yellow, dent kernels contain both hard and soft starch. The kernel can become indented at maturity causing the characteristic phenotype.

Flint corn *(Zea mays indurata)*, also known as Indian corn, is used for similar purposes as dent corn. Flint corn is distinguished by a hard outer shell and kernels with a range of colours from white to red. Silage maize in northern Europe is often of this type. The endosperm of waxy maize *(Zea mays var. ceratina)* almost exclusively contains amylopectin and hardly any amylose starch molecules in contrast to normal dent and flint maize varieties that contain both. Waxy maize contains up to 100% amylopectin (Campbell *et al*., 2002; Shi *et al*., 1998; Morrison *et al*., 1984). Waxy has a higher feeding value than genotypes higher in amylose (Svihus *et al*., 2005). High amylose (*Zea mays)* is opposite to waxy as it is high in amylose and has more than 50% amylose of total starch. It is also called amylomaize; a term coined by Robert P. Bear of Bear Hybrids Corn Company in Decatur, Illinois to describe his discovery and commercial breeding of this special corn starch. The discovery of amylomaize occurred as a mutation in a normal inbred line and from that one mutation, an entirely new kind of maize (Michalet-Doreau *et al*., 2004) was developed.

Maize genotypes can also differ in endosperm type, i.e. floury (dent) *vs.* horny (flint) (Kotarski *et al*., 1992; Michalet-Doreau and Champion, 1995). Dent maize starch is more loosely bound in a starch:zein protein matrix and becomes indented on maturity (Fox and Manley, 2009). Flint mostly has a thick, hard, vitreous endosperm layer surrounding a small, soft granular centre (Ettle *et al*., 2001). The relative amounts of soft and corneous starch, however, vary in different varieties. The vitreousness is the ratio of vitreous (hard) to floury (soft) endosperm (Fox and Manley, 2009), and is used to assess the type of maize endosperm. Therefore diverse genetic variations induce the diversity in starch of different maize genotypes and its feeding value.

Normal maize starch consists of 20-30% amylose and 70-80% amylopectin, whereas high-amylose maize contains >50% amylose and waxy maize contains almost 100% amylopectin (Morrison *et al*., 1984; Shi *et al*., 1998; Campbell *et al*., 2002). Amylose and amylopectin differ in crystallinity (Cheetham and Tao, 1998; Chen *et al*., 2006; Liu *et al*., 2009). These differences are relevant for kernel use as food and feed and for industrial application. For example, amylose and amylopectin

proportions determine the gelatinization temperature and influence the degradability by simple-stomached animals and ruminants (Van Hung *et al*., 2006).

Growth temperature

Maize is a relatively cold sensitive plant. Growth temperature significantly influences many physiological processes in maize, including photosynthesis, growth, development, morphology, production, quality and time necessary to reach maturity, with optimum temperatures usually around 30 ºC (Miedema, 1982; Struik and Stamp, 1985). Adverse growth temperature, either sub- or supra-optimal, may result in reduced whole-plant yield (Brooking, 1993) and reduced grain yield (Dale, 1983). Like many other developmental and growth processes, yield also depends on temperatures during a specific time span (Struik, 1983; Struik and Stamp, 1985, Struik *et al*., 1985).

During kernel development, i.e. the grain filing phase, growth temperature affects the extent to which the potential kernel size can be realized, because an increase in temperature enhances physiological activity. This results in an increased rate of grain filling and starch accumulation, but with shorter duration of grain filling by accelerating the rate of maturation (Bhullar and Jenner 1986; Wiegand and Cuellar, 1981). Any reduction in kernel growth and yield in maize at high temperatures is either due to an increase in rate of starch synthesis being inadequate to balance for a shorter growth period or reduction in both the rate of starch synthesis and reduced growth period. Due to this imbalanced effect of temperature on rate and duration of grain filling or kernel development, a higher temperature usually results in faster maturity but smaller final grain size. A higher growth temperature during grain filling not only reduces individual kernel weight but also the number of actively growing kernels (Tollenaar and Daynard, 1978; Struik, 1983; Fischer and Palmer, 1984; Cheikh and Jones, 1995; Andrade *et al*., 1996).

Starch accounts for most of the dry matter in maize grains; a reduction in final grain weight associated with unfavourable temperature during grain filing is largely due to a decrease in starch content (MacLeod and Duffus, 1988; Bhullar and Jenner 1986). These effects are mainly associated with changes in starch

biosynthesis (Monjardino *et al*., 2005). An increase in temperature during grain filling reduces the number and size of starch granules per kernel, affects the rate of starch accumulation (Jones *et al*., 1985; Commuri and Jones, 1999) but also the composition of starch, for example the chain length distribution (Lu *et al*., 1996).

Factors affecting rumen starch degradation in maize kernels, its rate and duration

Rumen starch degradation and genotypes

Starch degradation of maize kernels in rumen fluid is mainly influenced by the structure, composition and contents of the starch (Stevnebø *et al*., 2006). Starch structure and composition are influenced by maize genotype. Consequently, they differ in their degradation and feeding value (Frei, 2000; Troyer, 2000; Duvick, 2005). Rumen starch degradation can also be influenced by different types of endosperm in different maize genotypes. The variation in the rate and extent of starch degradation in the rumen due to genetic variation, therefore, plays an important role in determining the nutritive value of forage maize for ruminants.

Rumen starch degradation and growth temperature

Growth temperature, during kernel development, indirectly influences the starch degradation by influencing its biosynthesis and accumulation. It is known that not all enzymes involved in starch biosynthesis are equally sensitive to growth temperature. Soluble starch synthase is probably the most temperature sensitive enzyme. Soluble starch synthase activity depends on the temperature to which grains are exposed, it has a low temperature optimum for its activity and is also subject to heat inactivation, hence limiting starch synthesis under high temperature and depending on the duration of exposure to heat (Rijven, 1986; Hawker and Jenner, 1993; Keeling *et al*., 1993). Temperature also plays an important role in amylose and amylopectin proportions, with elevated temperature (35 ºC) reducing amylose content and the proportion of short-branch chains in amylopectin compared with lower temperature (25 ºC) (Lu *et al*., 1996).

The accumulation of starch in the kernels depends on growing conditions, especially growth temperature and maturity stage. Lower starch contents can be the result of either sub-optimal or supra-optimal temperatures during grain filling (Anker-Nilssen *et al*., 2006). Supra-optimal temperatures during grain filling can impede starch accumulation in combination with increased growth rate and a reduced grain-filling duration (Muchow, 1990). Supra-optimal temperatures may also impair starch synthesis (less starch per unit of endosperm, smaller starch granules) or change the composition (Tester *et al*., 1991; Tester *et al*., 1995) and hence can influence rumen degradation of starch. Sub-optimal temperatures result in less starch accumulation and lower starch contents by slower and limited grain filling, despite the advantage of longer growth duration (Muchow, 1990, Wilson *et al*., 1995).

Rumen starch degradation and oven-drying

As the standard technique to analyse rumen degradability uses dried and ground samples, differences in gelatination enthalpies (physical properties) are obscured by the drying method, as cows consume bruised maize in a moist form after ensiling, not in dried and ground. The feeding value measured in the dried samples most probably will not be the same as that of the fresh material. Rumen starch degradability in maize is also mainly affected by its physical characteristics and can be altered through any post-harvest processing (Yang *et al*., 2001). Post-harvest processing such as mechanical processing (Andrae *et al*., 2001) normally increases the degradability of starch (Yang *et al*., 2001). However, drying moist maize kernels (Allen, 2009) at high temperatures can also cause changes to its physical properties, by rearranging the amylose molecules (retro-gradation) and, thereby, decreasing its degradability (Rooney and Pflugfelder, 1986). Genotypes differing in starch structure and composition may show different responses to oven-drying, and hence the estimation of their feeding values may be influenced differently by sample processing (Haros and Suarez, 1997). Therefore there is a need to devise a method to measure *in vitro* rumen degradation of fresh maize kernels to evaluate its feeding value precisely. That could help breeders and feed modellers to

understand the impact of oven-drying on the feeding value and lead to redevelop feeding models.

Rumen starch degradation and maturity

Starch degradation can also by influenced by dry matter contents, i.e. maturity. Dry matter concentration (DM %) is a good descriptor of maturity (Jensen *et al*., 2005) and is an important tool to rank maize genotypes based on their maturity (Schwab *et al*., 2003; Marton *et al*., 2007). Maize genotypes differ in their maturity type and ripening (Tollenaar, 1989; Rebourg *et al*., 2003) and also in their response to growing temperatures. Maize genotypes can differ in nutritive value even at the same DM % (Hetta *et al*., 2012; Jensen *et al*., 2005). Such differences among genotypes make it difficult to understand how maize type and harvest date (maturity stage) influence starch accumulation or rate and extent of starch degradation (Ettle and Schwarz, 2003). Starch degradation can decrease with advancing maturity (Tolera *et al*., 1998; Tolera and Sundstøl, 1999; Ettle *et al*., 2001).

Maize silage feeding value, ensiling temperature and duration

Factors influencing feeding value of maize silage are shown in Fig. 3. Maize silage is an excellent source of energy and fibre for inclusion in the rations of ruminant livestock. It is well recognised for its ability to increase forage intake. In general, it improves rumen function and consequently leads to an overall improvement in animal condition. Maize is also very cost-effective forage to grow and feed. It is essential to have a good microbial fermentation process during ensiling, leading to a rapid decline in pH to about 4.0 to obtain stable silage (Oude Elferink *et al*., 2000). An ideal microbial fermentation process should result in a maximum conservation of dry matter, nutrients and energy with minimum nutritional losses. There are three kinds of nutritional losses associated with the ensiling process: silage making, storage and feed-out losses. Some of these losses are unavoidable like plant respiration losses (McGechan, 1990). Transportation

losses and fermentation losses and feed-out losses can be minimized by proper management of the silages.

An ideal fermentation process with rapid decline in pH (to about 4.0) not only depends on the type and quality of the forage crop, but also on the ensiling conditions (Oude Elferink *et al*., 2000). Ensiling temperature may affect the (relative) activity of the various microorganisms involved in the fermentation process. These microorganisms can be desirable or undesirable and both types play a vital role in ensiling fermentation and hence the feeding quality of the silage (Weinberg *et al*., 2001). These microorganisms can be divided into psychrophilic, mesophilic and thermophilic, according to their ability to grow at a low, moderate or high ensiling temperature, respectively.

Normally, active microbial processes in the silo causes a rapid decline in pH, and can continue up to about 2 to 6 weeks after ensiling, depending on the sugar content and ensiling conditions. Once the pH is around 4.0, the silage is stable for a long period until the silage is opened and exposed to air for feeding (Pahlow *et al*., 2003). Conversely, there is also evidence that some microbial activity occurs during the stable phase (Der Bedrosian *et al*., 2012). As such, ensiling duration may affect the final nutritional quality of maize silage. It is unknown how ensiling temperature and duration interact to influence the feeding quality of the maize silage.

Gas production system (brief description)

In this thesis, *in vitro* fermentation processes are used to study the feeding value of different samples with a fully automated, time related gas production system. By measuring the rate of gas production upon the fermentation of starch samples in buffered rumen fluid, the degradation kinetics of the starches can be analysed. The starch characteristics and *in vitro* ruminal degradation characteristics can be related to each other and differences in starch characteristics between maize genotypes and growth conditions of the maize identified.

Samples (0.5 g dry matter basis) are incubated in buffered rumen fluid, using the gas production technique as described by Cone *et al*. (1996). Rumen fluid is normally collected 2 h after the morning feeding from two rumen cannulated

lactating cows, fed to requirements. The samples are incubated in duplicate in 60 ml buffered rumen fluid in 250 ml bottles in a shaking water bath at 39 °C. Gas production is recorded for 72 h, using a fully automated system (Cone *et al*., 1996). Results are corrected for blank gas productions, i.e. gas production by buffered rumen fluid without a substrate. In addition, external standards can be run concurrently to allow batches of samples to be compared.

A gas production profile can be described with a tri-phasic model, each sub-curve is determined by an asymptotic maximum gas production (A), the time needed to reach half of A (B) and a parameter (C) determining the shape of the curve (Groot *et al*., 1996). The parameter B provides an indication of the rate of fermentation, a low value of B indicates a fast fermentation and a high value of B represents a slow fermentation. Cone *et al*. (1997) showed that the gas production of the first sub-curve (A1) is caused by fermentation of the soluble sugars and protein fraction, and that of the second sub-curve is caused by fermentation of the insoluble fraction i.e. insoluble carbohydrates (starch), neutral detergent fibre, etc. The third sub-curve is the result of microbial turnover after exhaustion of the substrate and is not related to feeding value (Cone *et al*., 1997). The observed gas production values were used to calculate starch degradation using the equation of Chai *et al*. (2004) (OM = organic matter):

$$\textit{Starch degradation at time t (g/kg OM)} = -191.6(\pm 14.6) + 0.303(\pm 0.025) \times \textit{Starch content (g/kg OM)} + 1.648(\pm 0.053) \times \textit{Gas produced at t (ml/g OM)}$$

Research questions

Starch degradation in maize kernels plays an important role in the feeding value of maize. The degradability of the starch in the animal can be influenced by both its chemical composition and the physical properties of the starch. Until now it is not known how the properties of starch in the kernels in the ear of the maize plant are influenced by maturation on the plant and by the genotype (dent, flint etc.). Nor it is known how maturation, cell wall degradability, chemical composition, physical properties and distribution of chemical components do interact with the properties of starch determining its degradability in the rumen.

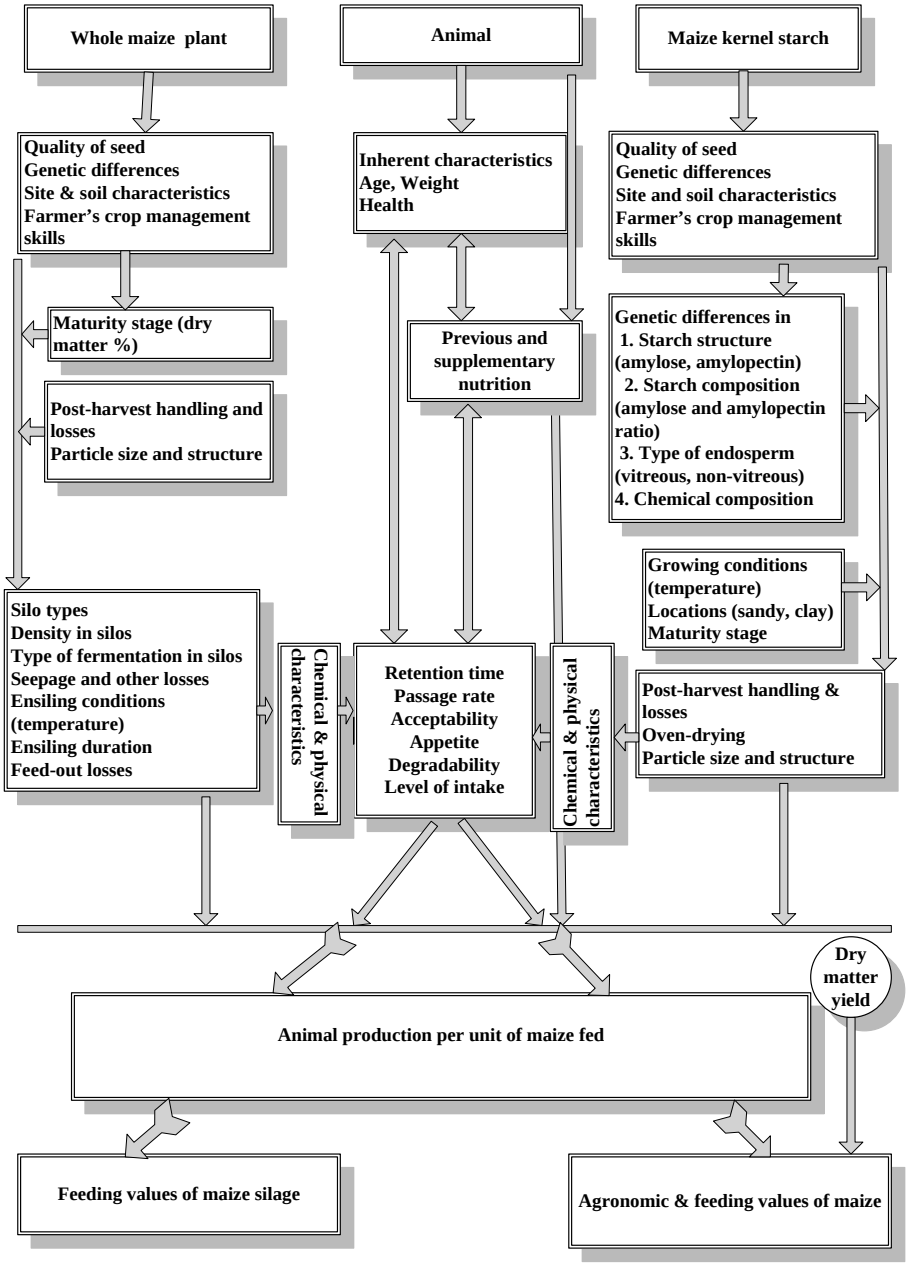

Fig. 3. Factors influencing feeding value of maize silage and agronomic and feeding value of maize starch.

Furthermore, it is unknown how the degree of ripening of starch (maturation) in the plant affects the degradability of starch in the maize kernels. Another complicating factor in this respect is that the physical properties of the starch are affected by the oven-drying, as oven-drying is normally conducted for the analysis of the chemical composition and the degradability. In addition, the interaction between the ripening of starch (maturity), chemical composition, physical properties and characteristics of the breed requires further investigation.

As starch degradability is mainly determined by its physical properties and as the differences in physical properties have most probably disappeared after drying the samples, new methods have to be developed to analyse the degradability of starch in fresh (un-dried) samples. Finally, for optimal ration composition, both the rate and extent of the degradation of the fresh starch samples have to be determined. This should also be done after ensiling the maize, as the animals eat the maize as silage, not in a freshly cut form. It is not clear how the ensiling process like ensiling temperature and duration influences the starch quality, hence feeding value of maize for dairy cattle.

Objectives

The major aim of the study is to gain insight into the relationships between plant properties, starch properties and rumen fermentation characteristics as well as insight into the factors determining starch degradability in maize. The final goal is to obtain tools for the maize breeders to allow genotypes to be bred with optimal degradable starch and to have tools to steer the starch quality. A “co-product” of the studies may be the development of methods to estimate the correct feed quality, including fermentation kinetics, of starch in maize silage.

In summary, the objectives of the work described in this thesis are:

1. To investigate the starch degradation of maize kernels as influenced by genotype, growing conditions and maturity stage, as measured with the gas production technique.

2. To determine the effect of temperature on the growth and development of kernel dry matter (starch accumulation) and dry weight per kernel in different genotypes of maize.
3. To determine the influence of oven-drying on the physical and anatomical properties of starch as well as on the degradability of starch as measured by the gas production technique.
4. To estimate the feeding value of maize silage for dairy cows as influenced by ensiling conditions particularly temperature and duration.

Structure of the thesis

A schematic illustration (Fig. 4) shows that this thesis consists of six chapters including this general introduction (**Chapter 1**). Chapters 2-5 represent the main body of the thesis reporting new studies to answer the above-mentioned research questions. These chapters have been written as stand-alone papers to be submitted to international peer-reviewed journals. **Chapter 2** investigates the influence of different maize genotypes and growing conditions on accumulation of dry matter at different maturity stages under controlled and field conditions. **Chapter 3** of this thesis reports a study on how maize genotypes (differing in starch content and composition) and growing conditions at specific maturity stages affect ruminal starch degradation and hence feeding value of starch in maize kernels. **Chapter 4** focuses on how the process of drying influences starch degradability in kernels of different maize genotypes grown under different conditions in lactating cows. In this study, only the process of drying was investigated while genotypes and growing conditions were same. A study investigating the nutritional value of maize silage as influenced by ensiling temperature and duration is reported in **Chapter 5**. Finally, **Chapter 6** provides a comprehensive discussion on the feeding value of starch in maize kernels for ruminants, especially lactating cows, and which factors influence the feeding value. This chapter also discusses how ensiling temperatures and duration affect the nutritional quality of maize silage.

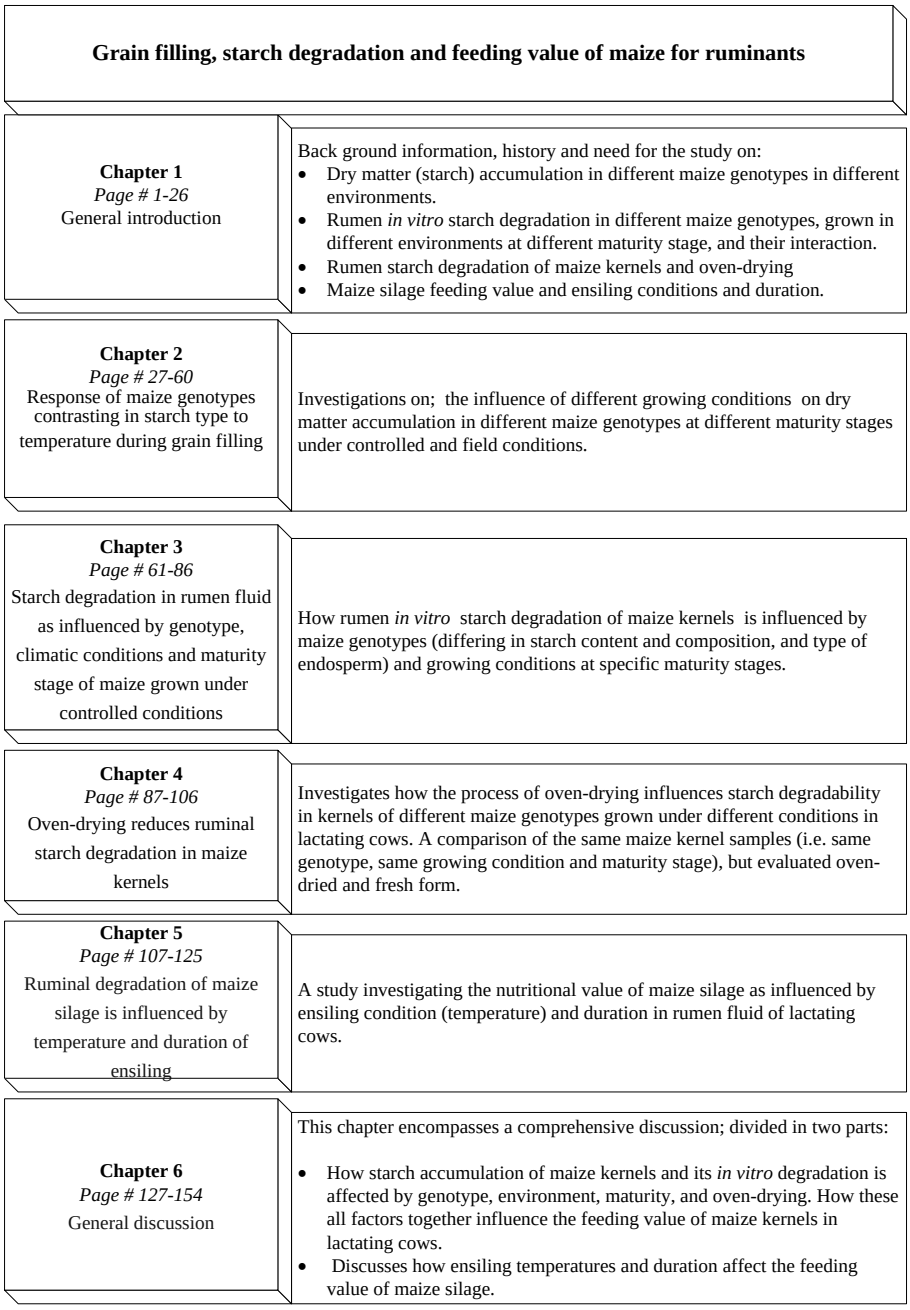

Fig. 4. A schematic illustration of structure of the thesis

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Response of maize genotypes contrasting in starch type to temperature during grain filling

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Chapter 2

ABSTRACT

Starch accumulation makes maize (*Zea mays* L) an important food, feed and fuel crop but its rate depends on environmental factors. We studied genetic variation in effects of environment and especially of temperature during grain filling, on genotypes contrasting in starch type and vitreousness. Six maize genotypes were grown in two climate-controlled greenhouse experiments under different day/night temperature regimes during grain filling (Exp. 1: 30/24, 24/18, 18/12 °C; Exp. 2: 32/22, 27/17, 22/12 °C) and harvested at six (Exp. 1) or four (Exp. 2) stages during grain filling. In four field experiments, another (narrow) set of six maize hybrids was grown on cover sand and river clay soils and harvested at six kernel maturity stages, whereas two hybrids contrasting in vitreousness were sown on three dates (before, at and after optimum sowing time) and harvested at five stages after silking. Moisture concentration declined with increasing thermal time during grain filling, and was lower for lower temperatures at the same thermal time. Maximum water content was reached at higher thermal time for medium temperatures than for lower and higher temperatures and decreased with an increase in temperature. The dent hybrid accumulated more water, whereas the waxy and its counterpart hybrids took less thermal time than the other hybrids to reach the maximum. Flint showed lower maximum water contents (mg/kernel) than other genotypes in both greenhouse experiments. Water contents (g/m^2) in the field experiments showed different trends with regard to soils but similar trends across genotypes across the years. Sandy soil showed higher maximum water contents than clay in the first year but in the second year, both showed similar maximum water contents. Non-vitreous genotypes showed higher and earlier maximum water contents than vitreous genotypes in both years. Kernel dry weight increased with thermal time but rate was slower at the highest temperature. The highest temperature also gave lower final kernel weight. Hybrids responded in a similar way to temperature treatments. In field experiments, patterns of change in moisture concentration and kernel dry yield were consistent across years, soil types, and sowing times, but not those in maximum water content; patterns in the field experiments reflected the patterns of the intermediate temperature of the greenhouse experiments most.

Keywords**:** Maize (*Zea mays* L.); grain filling; starch accumulation; starch mutants; temperature; hybrid.

1. Introduction

Maize (*Zea mays* L.) is an important grain crop used for food, feed and fuel (FAO, 2009). At maturity, it has high starch content (61% in kernel dry matter). Maize used as whole-plant silage is also major winter roughage for ruminants; it combines high energy content with good structural value due to its high starch content and highly digestible cell wall components in the stem (Deinum and Struik, 1989; Cone and Engels, 1993; Argillier *et al*., 1995; Cone *et al*., 2008).

Maize is a cold-sensitive C4 crop and many physiological processes and characteristics including photosynthesis, growth, development, morphology, production, starch accumulation, quality and time necessary to reach maturity are strongly affected by temperature, with optimum temperatures around 30 $^{\circ}$C (Miedema, 1982; Struik and Stamp, 1985; Kim *et al*., 2007). Sub- or supra-optimal temperatures are major causes of reduced whole-plant yield (Brooking, 1993) and reduced grain yield (Dale, 1983). Also diurnal temperature fluctuations affect whole plant dry matter yield of maize (Lu *et al*., 1996). As many developmental and growth processes are time specific, yield also depends on temperatures during a specific time span (Struik, 1983; Struik and Stamp, 1985). The effects of temperature on kernel growth and grain yield are mediated by the influence of other environmental factors, genotype, crop management and their interactions (Struik, 1983; Muchow, 1990; Brooking, 1993; Kim *et al*., 2007; Borrás and Gambín, 2010).

This paper focuses on the effects of temperature during grain filling, i.e. once the number of kernels and the potential kernel size has been set. During the grain filling phase, temperature affects the extent to which the potential kernel size can be realized, because an increase in temperature increases rate of grain filling and starch accumulation by enhancing physiological activity, but shortens the duration of grain filling by accelerating the rate of maturation in wheat and maize (Wiegand and Cuellar, 1981; Bhullar and Jenner, 1985, 1986; Kim *et al*., 2007; Borrás and Gambin, 2010). Since temperature effects on rate and duration are not balanced, a higher temperature usually results in faster maturity but smaller final grain size.

Starch accounts for most of the dry matter in maize grains and a reduction in final grain weight associated with unfavorable temperature during grain filling is

largely due to a decrease in starch content (see also Bhullar and Jenner, 1985, 1986 for wheat; see also MacLeod and Duffus, 1988 for barley). Therefore, any reduction in kernel growth and yield in maize at high temperature is either due to the increase in rate of starch synthesis being inadequate to balance for the shortened growth period or due to both a reduction in rate of starch synthesis and a shortened growth period due to high temperature. High-temperature stress may also affect protein accumulation (Monjardino *et al*., 2005).

An increase in temperature during grain filling reduces number and size of starch granules per kernel, affects the rate of starch accumulation (Jones *et al*., 1985; Commuri and Jones, 1999). It also decreases the total activity of the starch branching enzyme (Martínez *et al*., 2013). Increased temperature also affects the composition of starch, for example the chain length distribution (Lu *et al*., 1996), the AM to starch ratio (Martínez *et al*., 2013), or the AM and AP proportions (Lu *et al*., 1996). Elevated temperature (35 ºC) reduces AM content and the proportion of short-branch chains in AP compared with lower temperature (25 ºC) (Lu *et al*., 1996).

Starch biosynthesis is directly influenced by temperature. Studies show that not all enzymes involved in starch biosynthesis are equally sensitive to temperature. Soluble starch synthase (SSS) is probably the most temperature sensitive enzyme. SSS activity depends on the temperature to which grains are exposed, it has a low temperature optimum for its activity and is also subject to heat inactivation, hence limiting starch synthesis under high temperature depending on the duration of exposure to heat (Rijven, 1986; Hawker and Jenner, 1993; Keeling *et al*., 1993).

In maize, there is large genetic variation in starch structure and composition (Wilson *et al*., 2004), partly associated with the occurrence of many starch mutants, including waxy and high amylose. Normal starch consists of 30% amylose (AM; low level of branching) and 70% amylopectin (AP; highly branched), whereas high amylose maize contains 40-70% AM and waxy maize contains 100% AP (Morrison *et al*., 1984; Shi *et al*., 1998; Campbell *et al*., 2002). AP has 1000 times higher molecular weight than AM (Oates, 1997). AM and AP also differ in crystallinity (Cheetham and Tao, 1998; Chen *et al*., 2006; Liu *et al*., 2009). These differences are

relevant for grain use as food and feed and for industrial applications. For example, AM and AP proportions determine the gelatinization temperature and influence degradability by monogastrics and ruminants (Van Hung *et al.*, 2006). There are also large differences among genotypes in vitreousness, i.e. the ratio between hard and soft endosperm (Ettle *et al.*, 2001).

Most of the data on the impact of temperature on grain filling of maize hybrids are based on experiments in which temperature during grain filling is varied by varying sowing dates. The present study aims to understand how temperature during grain filling affects kernel dry matter accumulation in maize genotypes differing in starch composition and structure under controlled greenhouse conditions and compares these results with field data of different hybrids grown in different years, on different soils and with different sowing times.

2. Materials and methods

We carried out two greenhouse experiments and four field experiments with two or six contrasting hybrids, depending on the experiment (Table 1). Hybrids will be indicated by codes upon request of funding agencies. The genotypes in the greenhouse experiments were selected on the basis of variation in amylose content and type of endosperm. Four genotypes (dent, flint, high amylose and waxy) were present in both greenhouse experiments. In the first greenhouse experiment, also the normal counter parts of the high amylose and waxy genotypes were used while in the second greenhouse experiment also non-vitreous and vitreous endosperm (rumen escaping) types were used. The high amylose counterpart and the waxy counterpart had both similar amylose contents but were from different inbred lines. In the four field experiments, hybrids commonly grown in North-West Europe were used; they reflected a range of vitreousness.

2.1. Greenhouse experiments

Two pot experiments, coded Exp. 1 and Exp. 2, were conducted in the temperature controlled greenhouses of the Wageningen University Research Facility

Unifarm, Wageningen, the Netherlands, in the spring and summer seasons of the years 2008 and 2009. The genotypes used differed in earliness (ranging from FAO170 until FAO300) and were sown on different dates to get uniform silking time for all genotypes; sowing took place between 26 February and 4 March 2008 (Exp. 1) and between 18 March and 30 March 2009, later genotypes being sown earlier.

The fraction of incoming irradiance transmitted to the plant level was about 0.6 for photosynthetically active radiation (PAR). During daytime, supplemental light from 400 W SON-T Agro Philips (Philips, Eindhoven, The Netherlands) lamps (0.5 lamps m^{-2}; Exp. 1) or 600 W HPS Hortilux Schréder (Hortilux Schréder, Monster, The Netherlands) lamps (0.4 lamps m^{-2}; Exp. 2) was provided automatically as soon as global solar radiation outside the greenhouses dropped below 400 W m^{-2}. These lamps were switched off again when natural light exceeded 500 W m^{-2}. In Exp. 1, the day length was restricted to 12 h; and the day/night temperatures were set at 20/15 °C (12h/12h) until anthesis. In Exp. 2 it was not possible to restrict the day length; day length increased from about 12 h at emergence until about 15 h at anthesis. Until flowering the day/night temperatures were set at 21/16 °C (15h/9h).

After pollination, plants were allocated to three temperature treatments, imposed in separate greenhouses. In Exp. 1 these were 18/12 °C, 24/18 °C and 30/24 °C, in Exp. 2 these were 22/12 °C, 27/17 °C and 32/22 °C. Temperatures were slightly different between experiments as facilities for Exp. 1 had larger cooling capacity (with forced ventilation using cool air) during day time than in Exp. 2 (cooling through cold water in pipe system) and because preliminary analysis of the results of Exp. 1 suggested that desired treatment contrasts could be better realized with slightly higher temperatures.

Maize plants were grown in 12-L pots containing potting soil mixed with additional N, P, and K fertilizer. Total amounts of available N, P, and K per pot were 8.9, 2.5, and 14.2 g, respectively (Exp. 1) and 5.0, 1.5 and 8.0 g, respectively (Exp. 2), well above the amounts required for optimal growth. Two seeds were sown per pot at 4 cm depth; thinning to one plant per pot was done 1 week after

emergence. Plant density was 9 plants per m^2 in Exp. 1 and 6 plants per m^2 in Exp. 2. Plants were self-pollinated by hand and cross pollination was avoided by covering the ears. Silking and pollination dates were recorded as they formed the basis for the harvesting schemes.

Actual temperatures during grain filling were recorded to assess thermal time accurately. Thermal time was calculated with a base temperature of 8 °C (Wilkens and Singh, 2003). The base temperature is the temperature below which kernel growth is assumed to be zero. Accumulated thermal time was based on temperature data obtained from data loggers and was calculated as follows:

$$\textit{Accumulated Thermal Time (in °Cd)} = \Sigma\,[(T_{max} + T_{min})/2 - T_{base}]$$

where Tmax and Tmin are daily maximum and minimum temperatures and Tbase is base temperature, in °C.

At six (Exp. 1) or four (Exp. 2) dates after pollination with regular thermal time intervals, 50-125 kernels were harvested from one plant (Exp. 1) or from two plants (Exp. 2). The harvested kernels were separated into two different subsamples. One sub-sample was stored fresh for later analysis and one sub-sample was oven dried at 70 °C for 48 h. After 48 h, kernels were taken out from the oven and reweighed. We assessed fresh and dry weight per kernel and, based on these, kernel moisture concentration and content. Since we were only interested in a range of maturities relevant for harvesting of maize as silage, sampling was continued until physiological maturity of the grains, i.e. until the water content in the kernels had dropped until about 40%.

2.2. Field experiments

Four field experiments, Exps 3-6, were conducted on cover sand (‘sand’) and river clay (‘clay’) sites in the vicinity of Wageningen in the years 2008 and 2009. In all four field experiments, recommended doses of nutrients were applied

Table 1

Details on hybrids used in the six experiments, their symbols used in the graphs, and their presence in the experiments; G stands for genotype, CP = counterpart

Genotype (G)	Characteristic	Marker[1]	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
G1	Homozygous dent	○	X	X				
G2	Homozygous flint	□	X	X				
G3	High amylose	△	X	X				
G4	High amylose CP	▽	X					
G5	Waxy CP	◇	X					
G6	Waxy	⬡	X	X				
G7	Non-vitreous	◺		X	X	X	X	X
G8	Rather non-vitreous	▽			X	X		
G9	Medium non-vitreous	▭			X	X		
G10	Medium vitreous	⬠			X	X		
G11	Rather vitreous	⧖			X	X		
G12	Vitreous (rumen escaping)	◇		X	X	X	X	X

[1] Different fill for markers (when present in the figures) indicate low temperature (open), middle temperature (grey) or high temperature (black) or indicate clay (grey) or sand (black), or indicate early (open), mid-term (grey) or late (black) sowing time depending on experiment.

based on soil sampling. Weeds were chemically controlled shortly after emergence. Plant density was 9.5 (Exp. 3 on sand and Exp. 4 on clay in 2008) or 10.5 (Exp. 5 on sand and Exp. 6 on clay in 2009) m^{-2}. In Exps 3 and 4, six varieties (G7 – G12; Table 1) were sown in a randomized complete block design in four replications on May 6 and grown using recommended cultural practices. Individual plot dimensions were 15 m × 9 m. Choice of varieties was done based on the advice of the supervisory research committee and included varieties commonly grown in North-West Europe but differing in starch quality.

Accumulated thermal time was based on temperature data obtained from the Wageningen University Weather Station and calculated as indicated for the greenhouse experiments.

The crops of Exps 3 and 4 were sampled six times, bi-weekly during grain filling starting 14 days after silking by harvesting 2 rows of 1 m (1.5 m^2) per plot for the intermediate harvests while keeping two guard rows between samplings. Final harvest was carried out on 2 rows of 5 m length (7.5 m2). In Exps 5 and 6 varieties G7 and G12 (Table 1) were sown in a split-plot design with three replications on three different dates (April 20, May 8 and May 25); sowing date was the main factor and variety was the split factor. Individual plot dimensions were 13 m × 9 m. The crops of Exps 5 and 6 were sampled five times, bi-weekly during grain filling starting 14 days after silking by harvesting 1 rows of 1 m (0.75 m^2) per plot for the intermediate harvests while keeping two guard rows between samplings. Final harvest was carried out on 1 rows of 4 m length (3.0 m^2).

In all field experiments harvesting was done manually by picking the ears, counting them, shelling the cobs, and subsampling kernels and cobs to assess moisture concentration. Moisture concentration was determined after drying at 70 ºC for 48 h.

2.3. Detailed data analysis

For data analysis we used the conceptual framework of Borrás *et al*. (2009), but limit the number of traits presented in this paper. We will characterize the grain-filling patterns of the different combinations of grain-filling temperature and genotype using the development over thermal time of the traits moisture concentration (% water in total fresh weight), grain water content per kernel (mg) or per unit area (g/m^2), and kernel dry matter per kernel (mg) or per unit area (g/m^2).

Moisture concentration, water content and kernel dry matter were regressed against thermal time with the SAS software package. Moisture concentration (%, for Exps 1 to 6) was logistically regressed using the following equation:

$$Water\%t = Water\%min + (Water\%max - Water\%min)/(1+EXP(k\times(T-TM)))$$

where Water%t = moisture concentration (in %) at thermal time t; Water%min = minimum water content (in %); Water%max = maximum water content (in %); k = curve shape factor (no dimension); T = thermal time (in °Cd); TM = thermal time at which Water%t reaches 0.5 × (Water%max + Water%min) (in °Cd).

Maximum water content in all experiments (in mg per kernel or g/m^2) was assessed using the following 2nd order regression equation:

$$Water\ contents = a + b(tt) + c(tt)^2$$

where

a = intercept;

b and c = coefficients;

tt = accumulated thermal time in °Cd.

Kernel dry weight (mg, Exps 1 and 2) or final kernel yield (g/m^2, Exps 3 to 6) was logistically regressed using the following equation:

$$(Kernel\ dry\ weight\ or\ yield)t = (Weight\ or\ yield)max/(1+EXP(-k\times(T-TM)))$$

where (Kernel dry weight or yield)t = kernel dry weight (mg) or yield (g/m^2) at thermal time t; (Weight or yield)max / = maximum kernel dry weight (mg) or yield (g/m^2); k = curve shape factor (no dimension); T = thermal time (in °Cd); TM = thermal time at which (Kernel dry weight or yield)t reaches 0.5 × (Weight or thermal time at which (Kernel dry weight or yield)t reaches 0.5 × (Weight or yield)max (in °Cd).

For the greenhouse experiments, the impact of growth temperature and genotype on final kernel weight was analyzed in SAS software using following equation of GLM procedure:

$$Y_{ijk} = \mu + G_i + T_j + R_k + G_i \times T_j + G_i \times R_k + T_j \times R_k + \varepsilon_{ijk}$$

where

Y_{ijk} = final kernel weight (mg)

μ = overall mean;

G_i = genotype;

T_j = temperature;

R_k = replication;

ε_{ijk} = general error term.

Data on kernel yield/m^2 in the field experiments were analyzed using the non-linear analysis of variance model in SAS software by using following equation:

Exps 3 and 4

$$Y_{ijk} = \mu + G_i + S_j + R_k + G_i \times S_j + G_i \times R_k + S_j \times R_k + \varepsilon_{ijk}$$

where

Y_{ijk} = kernel yield (g/m^2)

μ = overall mean;

G_i = genotype;

S_j = soil;

R_k = replication;

ε_{ijk} = general error term.

Exps 5 and 6

$$Y_{ijk} = \mu + G_i + S_j + ST_k + R_l + G_i \times S_j + G_i \times ST_k + G_i \times R_l + S_j \times ST_k + S_j \times R_l + T_i \times R_k + ST_j \times R_k + G_i \times S_j \times ST_k + S_j \times ST_k \times R_l + \varepsilon_{ijkl}$$

where

G_i = genotype;

S_j = soil type (clay or sand);

ST_k = sowing time;

R_l = replication;

ε_{ijkl} = general error term.

3. Results

3.1. Greenhouse experiments

3.1.1. Moisture concentration

Figure 1 shows the relationship between moisture concentration and accumulated thermal time for both experiments. The R^2 values for curves of moisture concentration against thermal time were usually 99% or higher (data not shown). At the same thermal time the lower temperatures usually had lower moisture concentrations than the other temperatures, especially in Exp. 2. Higher temperatures also accumulated more thermal time during grain filling until physiological maturity, especially in Exp. 2. Moisture concentration at the same thermal time was higher for the dent and high amylose types than for the other types in both experiments (Fig. 1).

Final moisture concentration differed significantly among genotypes and temperature treatments ($P<0.0001$ for genotype, temperature and interaction effects in both experiments). There were also genotype × temperature interactions: some genotypes (e.g. flint G2 and waxy G6) did not respond strongly to temperature, whereas other genotypes (e.g. the dent G1 and the high amylase G3) showed a strong response consistent across the two experiments. Given the very different development over thermal time, an analysis of the final values is physiologically not meaningful.

3.1.2. Water content

Figure 2 shows the water contents per kernel against thermal time. In both experiments, the three temperature treatments showed distinctly different patterns with a narrower range and an earlier peak for the lower temperatures. In both experiments, maximum water contents were usually higher for the lower temperatures than for intermediate and higher temperature regimes (Tables 2 and 3). The dent hybrid (G1) accumulated more water, the waxy hybrid (G6) and its counterpart (G5) showed lower maximum water content than the other hybrids. Flint (G2) took less thermal time to reach maximum water content (Tables 2 and 3). The

maximum water contents were achieved between 300 to 500 °Cd (Fig. 2, Tables 2 and 3).

3.1.3. Kernel dry weight

Figure 3 shows the relationship between kernel dry weight and accumulated thermal time for both experiments. The R^2 values for curves of kernel weight against thermal time were usually 98% or higher (data no shown). In both experiments, the increase in dry weight per unit increase in thermal time was lower for the high temperatures than for the other temperatures; the highest temperatures also required more thermal time to level off than the other temperatures in both experiments (Fig. 3). Figure 3 showed very little variation in behavior among hybrids.

At the end of the experiments, individual kernel weights differed significantly among genotypes and temperature treatments ($P<0.0001$ for genotype, temperature in both experiments) (Tables 2 and 3). The interactions between genotype and temperature were not significant. The intermediate temperature gave the highest individual kernel weight in both experiments, whereas the highest temperature resulted in lowest kernel weights (Tables 2 and 3). The normal hybrids G1 and G6 gave the highest values among the hybrids in both experiments (Tables 2 and 3).

3.2. Field experiments

3.2.1. Moisture concentration

Figure 4 shows the relationship between moisture concentration and accumulated thermal time for all four field experiments. The R^2 values for curves of moisture content against thermal time were usually 99% or higher (data not shown). Moisture concentration showed a typical pattern: the initial decrease was slow, followed by a rapid decline until values below 40% after which the further decrease was slow again (Fig. 4), but the effects of year, soil, sowing time and hybrid on the patterns were negligible.

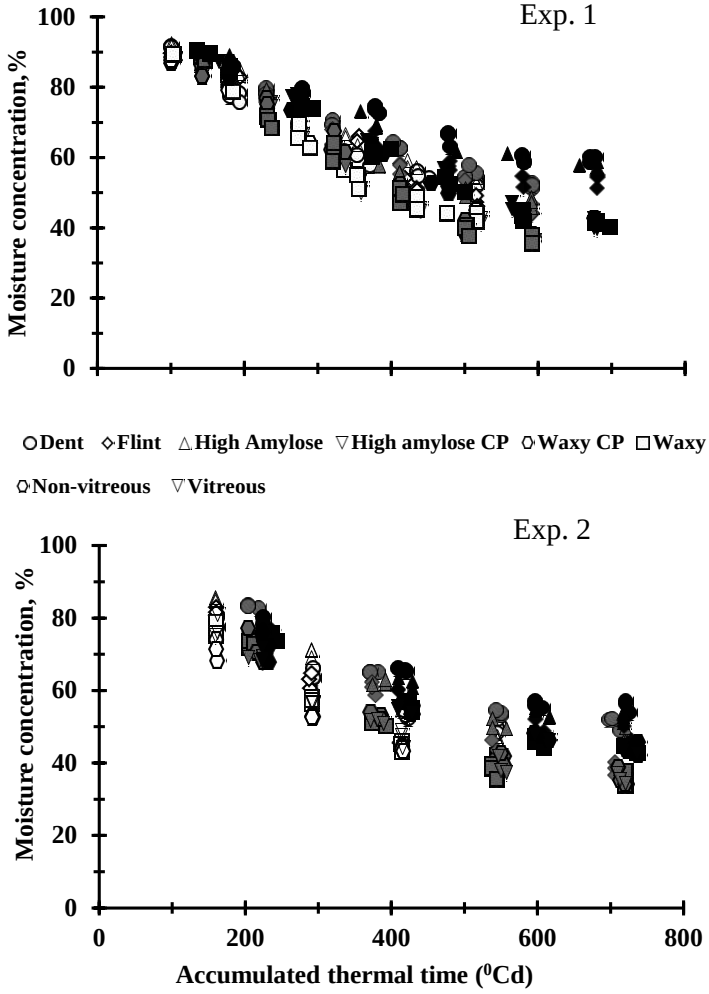

Fig. 1. Moisture concentration in the kernels (in % of fresh weight) against thermal time (in °Cd; base temperature 8 ˚C) in Exp. 1 (upper panel) and Exp. 2 (lower panel). Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 ºC in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 ºC in Exp. 2) and high (30/24 in Exp. 1 and 32/22 ºC in Exp. 2) temperature treatments during grain filling, respectively.

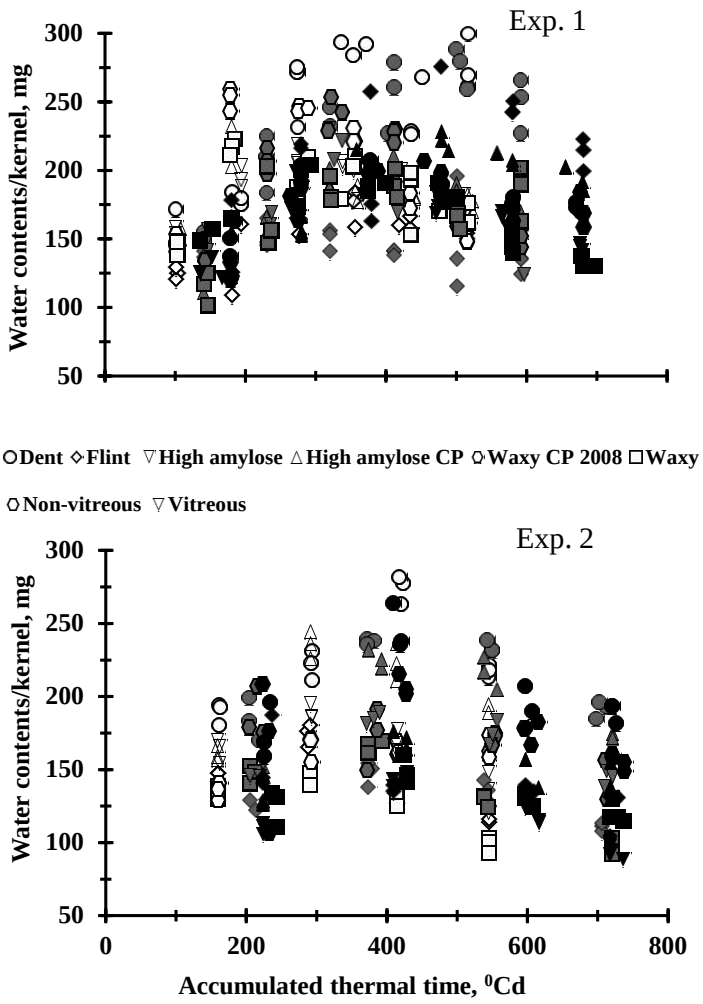

Fig. 2. Water content in the kernels (in mg) against thermal time (in °Cd; base temperature 8 ˚C) in Exp. 1 (upper panel) and Exp. 2 (lower panel). Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 ºC in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 ºC in Exp. 2) and high (30/24 in Exp. 1 and 32/22 ºC in Exp. 2) temperature treatments during grain filling, respectively.

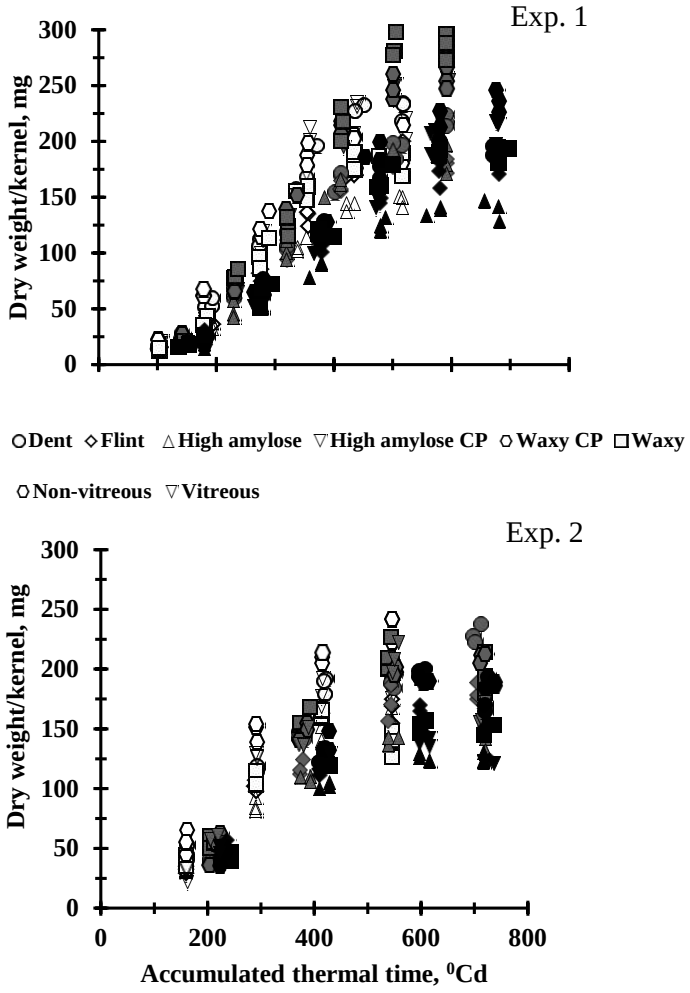

Fig. 3. Kernel dry weight against thermal time (in °Cd; base temperature 8 ˚C) in Exp.1 (upper panel) and Exp. 2 (lower panel). Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 ºC in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 ºC in Exp. 2) and high (30/24 in Exp. 1 and 32/22 ºC in Exp. 2) temperature treatments during grain filling, respectively.

3.2.2. Water content

Figure 5 shows the water contents in the kernels per unit area against thermal time. In all experiments the maximum values were obtained at about 300 °Cd. Water contents trend was similar for genotype but was different for soil types across the year (Table 4 and 5). Non-vitreous showed higher and earlier water contents than vitreous genotypes in both years. Sandy soil showed higher water contents than clay in the first year but in the second year, both showed almost same water contents. In Exps 5 and 6, the late sowing dates showed higher maximum water content than the earlier sowings. Whereas intermediate sowing time (S2) showed higher thermal time values for reaching maximum water contents (Table 5). Genotype was the dominant factor in determining the maximum water amount of water per unit area, with a consistent, large difference between G7 and G12 (Tables 4 and 5).

3.2.3. Kernel dry matter yield

Figure 6 shows the relationship between kernel dry weight per unit area and accumulated thermal time for all four field experiments. The R^2 values for curves of kernel yield against thermal time were usually 97% or higher (data no shown). Scatter (the variation) was very small during early stages of grain filling, but increased over thermal time. Tables 4 and 5 illustrate that soil type, genotype, and sowing time all had significant effects on kernel yield per unit area, but interactions were not significant. The sandy soil gave higher yields than the clay soil and the earlier the sowing time the higher the kernel yield; the kernel yields were relatively lower for G9, than for G11 and G12 (Tables 4 and 5).

Table 2
Maximum water content (mg/kernel) and final kernel dry weight (mg/kernel) for the temperature and genotype combinations of Exp. 1. The temperature × genotype interaction was not statistically significant.

Temperature (T), °C day/night	Genotype (G)	Maximum water content*, mg	Thermal time at maximum*, °Cd	Final kernel weight, mg	
				Mean	SEM
18/12	Dent	311	434	160.6	6.01
	Flint	167	400	154.1	7.58
	High amylose	231	362	150.7	9.81
	High amylose CP	201	311	179.3	7.94
	Waxy CP	189	301	172.4	8.95
	Waxy	202	306	174.0	11.46
24/18	Dent	265	499	202.8	5.24
	Flint	165	383	193.4	8.30
	High amylose	218	409	186.3	2.78
	High amylose CP	194	362	211.7	2.41
	Waxy CP	181	330	209.3	4.75
	Waxy	190	441	211.6	10.94
30/24	Dent	201	440	144.1	2.41
	Flint	143	321	139.0	5.95
	High amylose	179	393	130.5	1.20
	High amylose CP	185	406	166.0	5.22
	Waxy CP	176	339	166.5	0.95
	Waxy	177	426	167.8	5.14
P-value					
T				<.0001	
	18/12	217	352	165.2[b]	
	24/18	202	404	202.5[a]	
	30/24	177	387	152.3[c]	
G				<.0001	
	Dent	259	458	169.2[b]	
	Flint	158	368	162.2[c]	
	High amylose	209	388	155.8[d]	
	High amylose CP	193	360	185.7[a]	
	Waxy CP	182	324	182.7[a]	
	Waxy	189	391	184.4[a]	

Means followed by different letters are significantly (P<0.05) different.
CP: Counterpart.
* Estimates based on curve fitting.

Table 3
Maximum water content (mg/kernel) and final kernel dry weight (mg/kernel) and for the temperature and genotype combinations of Exp. 2. The temperature × genotype interaction was not statistically significant.

Temperature (T), °C day/night	Genotype (G)	Maximum water content*, mg	Thermal time at maximum*, °Cd	Final kernel weight, mg	
				Mean	SEM
22/12	Dent	259	404	181.1	4.91
	Flint	161	246	169.8	3.13
	High amylose	236	363	165.0	6.21
	Waxy	223	345	189.0	4.03
	Non-vitreous	218	332	181.3	3.61
	Vitreous	182	304	178.6	3.56
27/17	Dent	239	460	210.3	2.51
	Flint	144	417	201.7	1.16
	High amylose	227	473	189.3	11.14
	Waxy	218	402	219.5	8.22
	Non-vitreous	177	323	205.8	4.85
	Vitreous	180	448	197.9	11.09
32/22	Dent	226	470	158.9	7.70
	Flint	125	385	151.5	8.82
	High amylose	225	418	138.5	6.51
	Waxy	198	245	174.0	2.33
	Non-vitreous	203	372	171.0	3.12
	Vitreous	178	338	170.6	1.00
P-value					
T				<.0001	
	18/12	213	332	177.5[b]	
	24/18	198	420	204.1[a]	
	30/24	193	371	160.8[c]	
G				<.0001	
	Dent	242	444	183.4[c]	
	Flint	143	349	174.3[d]	
	High amylose	229	418	164.3[a]	
	Waxy	213	331	194.2[b]	
	Non-vitreous	200	342	186.0[b]	
	Vitreous	180	363	182.3	

Means followed by different letters are significantly (P<0.05) different.
* Estimates based on curve fitting.

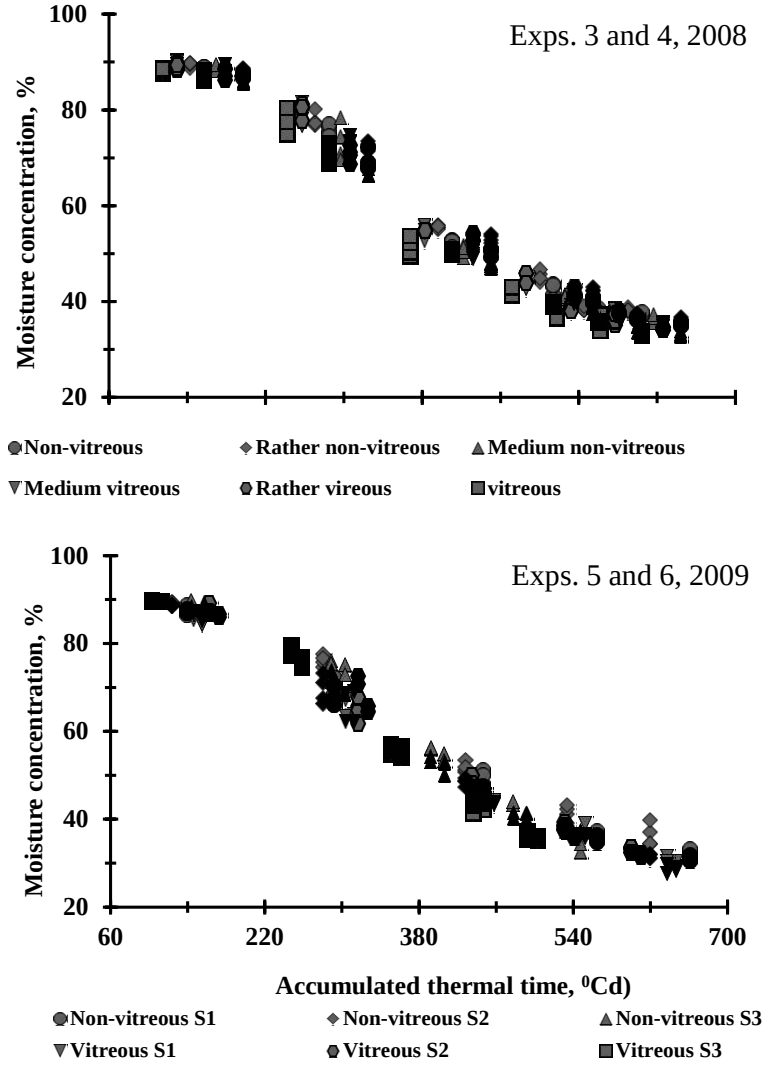

Fig. 4. Moisture concentration in the kernels (in % of fresh weight) against thermal time (in °Cd; base temperature 8 ˚C) in Exps 3 and 4 (upper panel) and Exps 5 and 6 (lower panel). Grey and black markers are for clay and sand, respectively.

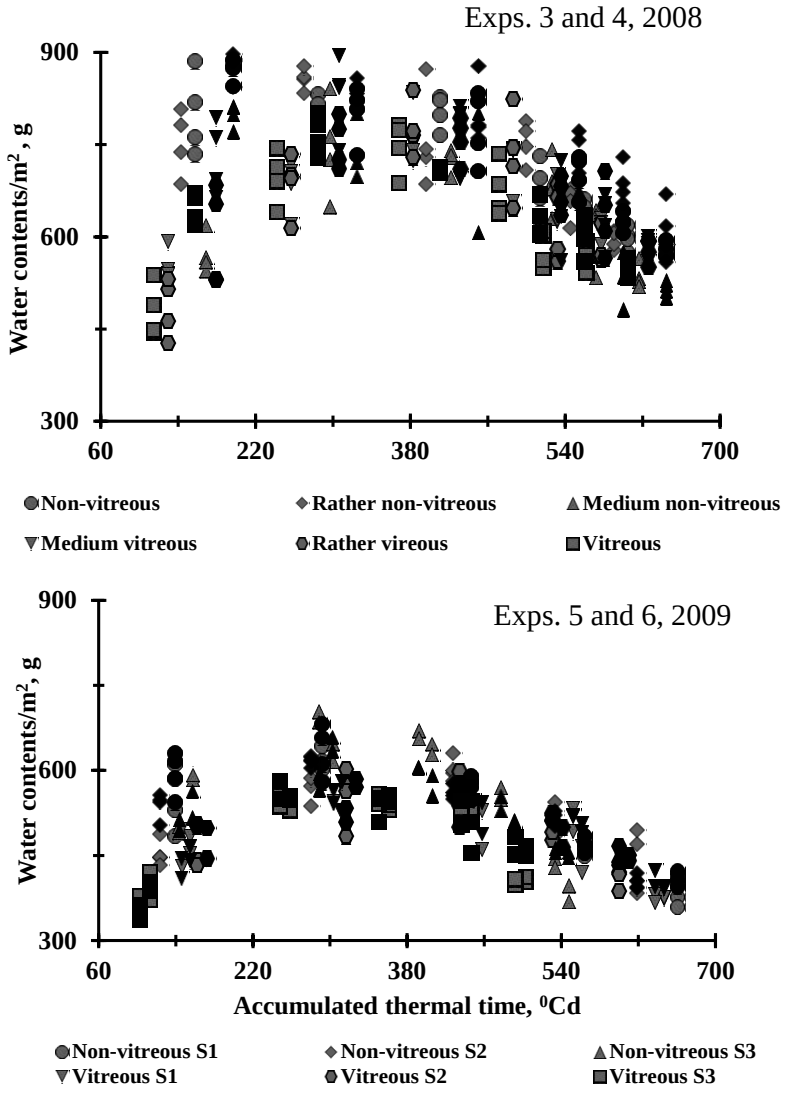

Fig. 5. Water content in the kernels (in g/m^2) against thermal time (in °Cd; base temperature 8 ˚C) in Exps 3 and 4 (upper panel) and Exps 5 and 6 (lower panel). Grey and black markers are for clay and sand, respectively.

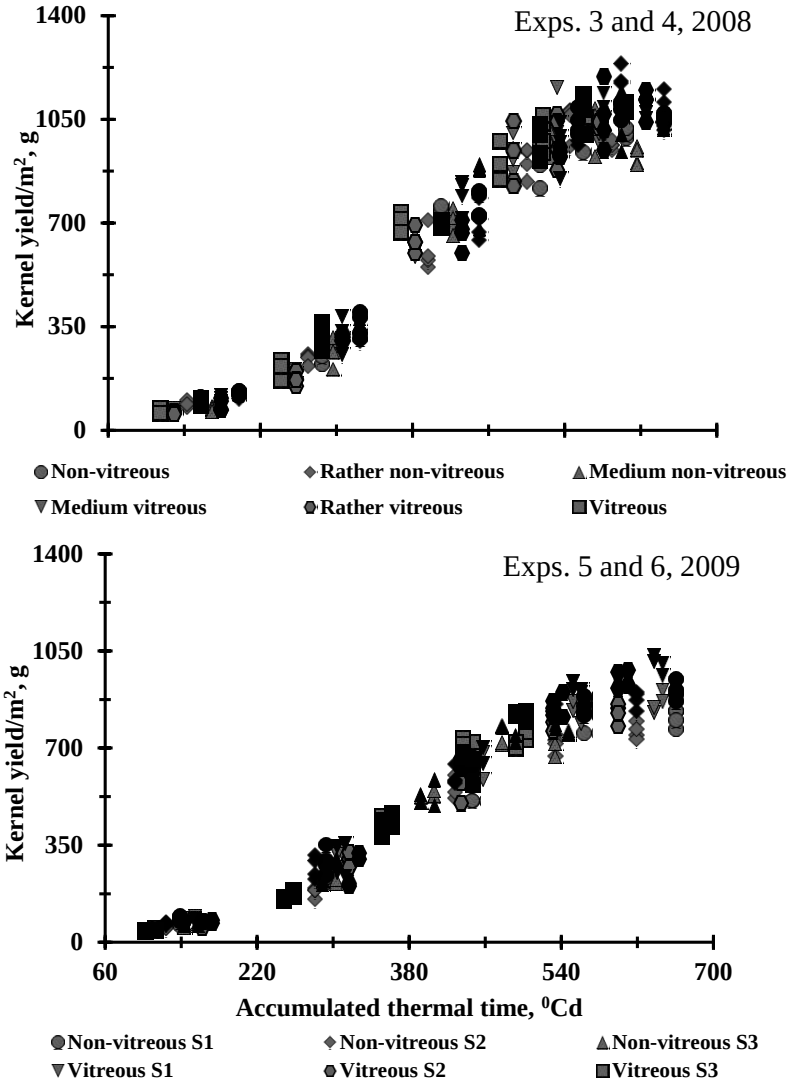

Fig. 6. Dry matter kernel yield (in g/m^2) against thermal time (in °Cd; base temperature 8 ˚C) in Exps 3 and 4 (upper panel) and Exps 5 and 6 (lower panel). Grey and black markers are for clay and sandy soil respectively.

Table 4
Maximum water content (g/m^2) and final kernel dry yield (g/m^2) for the soil type and genotype combinations of Exp. 3 and 4.

Experiment	Soil	Genotype (G)	Maximum water content*, g/m^2	Thermal time at maximum*, °Cd	Final kernel yield, g/m^2	
					Mean	SEM
Exp. 3	Sand	Non-vitreous	860	167	1056	12.2
		Rather non-vitreous	874	227	1076	66.3
		Medium non-vitreous	816	304	1035	19.2
		Medium vitreous	802	329	1078	21.6
		Rather vitreous	763	390	1106	45.6
		Vitreous	740	329	1091	13.6
Exp. 4	Clay	Non-vitreous	829	274	1005	15.9
		Rather non-vitreous	825	298	964	13.1
		Medium non-vitreous	752	379	928	29.6
		Medium vitreous	748	376	996	17.6
		Rather vitreous	767	374	1033	10.3
		Vitreous	739	352	1027	31.8
P-value						
Soil type					<.0001	
		Sand	809	291	1073[a]	
		Clay	777	342	992[b]	
G					0.0002	
		Non-vitreous	845	220	1030[bc]	
		Rather non-vitreous	850	262	1019[c]	
		Medium non-vitreous	784	342	982[d]	
		Medium vitreous	775	352	1037[bc]	
		Rather vitreous	765	382	1070[a]	
		Vitreous	739	341	1059[ab]	

Means followed by different letters are significantly (P<0.05) different.
* Estimates based on curve fitting.

Table 5
Maximum water content (g/m^2) and final kernel dry yield (g/m^2) for the soil type genotype and sowing time combinations of Exp. 5 and 6.

Experiment	Soil	Genotype (G)	Maximum water content*, g/m^2	Thermal time at maximum*, °Cd	Final kernel yield, g/m^2	
					Mean	SEM
Exp. 5	Sand	Non-vitreous, S1	620	258	905	34.0
		Non-vitreous, S2	605	304	875	30.6
		Non-vitreous, S3	613	318	768	14.4
		Vitreous, S1	546	367	1001	31.1
		Vitreous, S2	563	365	949	33.6
		Vitreous, S3	559	328	818	18.3
Exp. 6	Clay	Non-vitreous, S1	589	304	809	31.1
		Non-vitreous, S2	599	362	761	28.8
		Non-vitreous, S3	677	315	727	41.1
		Vitreous, S1	537	349	861	33.7
		Vitreous, S2	562	366	827	35.1
		Vitreous, S3	557	314	727	24.7
P-value						
Soil type					<.0001	
		Sand	584	324	886[a]	
		Clay	587	335	785[b]	
Sowing time					<.0001	
		S1	573	320	894[a]	
		S2	582	349	853[b]	
		S3	602	319	760[c]	
G					<.0001	
		Non-vitreous	617	310	808[b]	
		Vitreous	554	348	864[a]	

Means followed by different letters are significantly (P<0.05) different.
* Estimates based on curve fitting.

4. Discussion

4.1. General interpretation of observed effects

On the basis of both the greenhouse experiments and the field experiments (especially the ones in which sowing time was a factor), we can conclude that thermal time and temperature consistently influenced moisture concentration, water content, individual kernel weight and kernel yield (Fig. 1, 2 and 3). Our data indicate that both kernel growth rate and final kernel size are influenced by temperature and that at the same thermal time different temperature regimes will result in different values of moisture concentration, water content and dry kernel weight (Fig. 1, 2 and 3). Our data also show that slower growth rate of kernels per unit of thermal time is associated with lower final yields (Fig. 3; Tables 2 and 3).

In the greenhouse experiments with diverse temperature regimes, different genotypes showed similar responses to temperature treatment for kernel dry matter (Fig. 3, Tables 2 and 3), but varied in their response to temperature for moisture concentration and water content. Some hybrids (e.g., G1 and G5) showed a strong temperature effect on maximum water content (Tables 2 and 3), with a large contrast between the highest temperature and the two other temperatures, whereas other hybrids (G3 (high amylose) and G6 (waxy) showed much smaller differences in water content among temperature treatments. In the field experiments, temperature effects were visible, for example through the effects of sowing time, but these were accounted for by thermal time. The pattern of the changes in moisture concentration, water content and kernel yield against thermal time in the field were very much in line with the responses shown in the greenhouse experiments. Hybrids grown in these experiments responded in a similar way to the various sources of environmental variation for all parameters described.

4.2. Temperature effects on kernel development and dry matter accumulation

Low dry matter accumulation at high temperature is due to the fact that high temperature reduces the number of actively growing kernels and individual kernel weight (Tollenaar and Daynard, 1978; Fischer and Palmer, 1984; Cheikh and

Jones, 1995; Andrade *et al*., 1996). We did not observe an effect of the temperature regimes on kernel number as these regimes were imposed after silking. However, kernel number is also a function of kernel abortion, which can be enhanced by high temperature (Struik, 1983; Cheikh and Jones, 1994; Cheikh and Jones, 1995). Abortion did not play a role in our greenhouse experiments.

After pollination, kernel weight is affected by temperature because of its effects on rate and duration of cell division in the endosperm (Jones *et al.*, 1985) and rate and duration of starch accumulation (Jones *et al*., 1985; Commuri and Jones, 1999) once the number of endosperm cells is fixed.

High temperature reduces the duration of grain filling by inactivation and restriction of metabolic introversion (Bhullar and Jenner, 1985; 1986) probably by reducing invertase activity (Cheikh and Jones, 1995). Invertase plays an important role in realizing the potential kernel size by sucrose hydrolysis to hexoses, i.e. the substrate for starch synthesis (Hanft and Jones, 1986; Cheikh and Jones, 1995). So, invertase is responsible for higher concentration of hexoses, required for cell division during the lag phase (Ou-Lee and Setter, 1985; Hanft and Jones, 1986). Kernel development is blocked at high temperature, when there is a shortage of invertase (Miller and Chourey, 1992; Cheng *et al*., 1996). So, low rate of dry matter accumulation at high temperature per unit of thermal time suggests that invertase activity is critical for maize kernel development.

4.3. Temperature effects on starch accumulation

High temperature slows down starch synthesis (Jenner, 1994) due to reduced hydrolysis of sucrose (Jenner, 1994; Commuri and Jones, 2001). We observed in the greenhouse experiments that the genotypes widely differing in starch structure and composition showed very similar responses to different temperature regimes for dry mater accumulation. We had expected larger differences among genotypes as temperature is reported to affect the starch synthesis pattern (Lu *et al*., 1996) due to differences in enzymes activities involved in starch synthesis (Keeling *et al*., 1993). Temperature also affects the AM and AP ratio in starch (Lu *et al*., 1996). Cheng *et al*. (2005) found that elevated temperature increased AM contents

of a high AM containing genotype and decreased AM contents of low AM containing genotype.

High rate of dry matter accumulation at high temperature per unit of time could be due to high GBSS activities, an enzyme responsible for AM synthesis. This enzyme shows high activity even at high temperatures up to 45 °C (Keeling *et al*., 1993). GBSS is a product of the waxy gene (Wang *et al*., 1995; Fujita *et al*., 2001; Nishi *et al*., 2001). However, we observed that the rate of dry matter accumulation was lower for the highest temperature treatment per unit of thermal time (Fig. 3). Differences between genotypes in their response to temperature can partly be due to genotypic differences in response of AM contents to temperature associated with genetic diversification in waxy genes or differences in expression of various GBSS isoforms (Cheng *et al*., 2005). The waxy genotype used in our experiments contains 100% AP. The enzymes involved in production of AP are SSS, SBE, SS1, SS2 and SS3 (Tomlinson and Denyer, 2003). The enzyme SSS is heat sensitive (Keeling *et al*., 1993) and its activities cease at 35 °C (Jenner, 1994). Even then producing a high kernel weight at high temperature as observed in our experiments makes sense. High temperature treatments used in our two experiments were within the optimum range of SSS (25 °C) and SBE (27.5 °C) and did not exceed 35 °C to limit the yield. Despite the large variation in AM and AP we did not observe clear genetic differences in kernel weight in response to temperature. The field experiments did not include enough variation in environmental factors to trigger large variation among the hybrids selected.

5. Conclusions

We observed strong effects of temperature and genotype on rate of dry matter accumulation and final weight of kernels of maize. The temperature regimes applied in our experiments did embrace the optimum temperatures for most enzymes involved in starch synthesis, but the highest temperature regimes were most likely below the ceiling temperatures for these enzymes and thus did not block starch synthesis. We did not observe strong interactions between temperature and genotype.

We conclude that different genotypes (based on starch structure and compositions) behave similarly to different temperature treatments, despite possible differences in temperature response of specific enzymes involved in the starch biosynthesis and accumulation pathways.

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Starch degradation in rumen fluid as influenced by genotype, climatic conditions and maturity stage of maize grown under controlled conditions

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Chapter 3

Submitted to Animal Feed Science and Technology

ABSTRACT

Starch is the major component of maize kernels, contributing significantly to the feeding value of forage maize when fed to ruminants. The effects of genotype, climatic conditions and maturity stage on starch content in the kernels and on *in vitro* starch degradability in rumen fluid were investigated. Kernels of six maize genotypes, differing in amylose content and vitreousness, grown under three contrasting day/night temperature regimes during grain filling and harvested at different maturity stages from two greenhouse experiments were investigated. Starch content was measured using an enzymatic method and the gas production technique was used to assess starch degradation in rumen fluid of dairy cows. The extent of starch degradation at different incubation times (6, 12 and 20 h) was calculated from measured gas production data (6, 12 and 20 h, respectively) and a published equation. Gas production (ml gas/g organic matter) showed a positive linear relationship with starch content in the kernels up to a certain level of starch accumulation. At each maturity stage, whole kernel and starch degradation in rumen fluid depended on the genotype ($P<0.0001$), growing conditions ($P<0.0001$), and starch content ($P<0.0001$) and starch amount ($P<0.0001$) in the kernels. While starch content increased with advancing maturity, starch degradation similarly increased up to a certain level of starch content. *In vitro* starch degradation of the maize kernels in rumen fluid was affected by the starch composition, e.g. amylose and amylopectin content. Starch degradation was inversely related to the amylose content and vitreousness. Higher starch degradation was observed in the waxy (no amylose) and non-vitreous genotypes. The highest starch degradation was observed when plants were grown at intermediate temperatures in both experiments. The difference in starch degradability of each genotype at the same accumulated thermal time, i.e. maturity stage, was due to differences in grain filling rate, caused by the different temperature regimes. This effect of genotype and climatic conditions was consistent for all incubation times ($P<0.0001$). Rumen *in vitro* starch degradation is significantly influenced by genotypic, differences in starch content of the maize kernels and their growing conditions.

Keywords: Maize (*Zea mays* L.), Rumen degradation, Starch, Growth temperature, Maturity stage, Feeding value.

1. Introduction

The feeding value of forage maize for ruminants largely depends on its starch content and rumen fermentation characteristics (Theurer, 1986; Canizares *et al*., 2011). Starch degradation of maize kernels in rumen fluid is mainly influenced by starch content, composition of the starch (amylose, amylopectin) and physical properties of the starch (Wolters and Cone, 1992; Stevnebø *et al*., 2006). Different maize genotypes have different starch structures (i.e. amylose or amylopectin) and composition (amylose: amylopectin) (Shannon, 1984). Starch is composed of two distinct polymers, amylose and the higher molecular weight amylopectin. Amylose is a linear polymer of glucose units, with α-1,4-linkages, whereas amylopectin is a highly branched polymer with α-1, 6-linkages next to the α-1,4-linkages (Jackson, 2003). Normal maize starch is a mixture of amylose (20-30%) and amylopectin (70-80%), but this can vary among genotypes (Fankhauser *et al*., 1989). Consequently, genotypes can differ in starch degradation in rumen fluid and, therefore, in feeding value (Frei, 2000; Troyer, 2001; Duvick, 2005; Cone *et al*., 2008). Maize genotypes can also differ in type of endosperm, i.e. floury (dent) *vs.* horny (flint) (Kotarski *et al*., 1992; Michalet-Doreau and Champion, 1995). Dent maize kernel starch is more loosely bound in a starch-zein protein matrix and becomes indented at maturity (Fox and Manley, 2009). Flint maize kernels mostly have a thick, hard, vitreous endosperm layer surrounding a small, soft granular centre (Ettle *et al*., 2001). The relative amounts of soft and corneous starch, however, vary among cultivars. The vitreousness is the ratio of vitreous (hard) to floury (soft) endosperm (Fox and Manley, 2009), and is used to assess the type of maize endosperm. The variation in rate and extent of maize starch degradation in the rumen due to genetic variation, therefore, plays an important role in determining the nutritive value of forage maize for ruminants (Cone *et al*., 2008).

The accumulation of starch in the kernels depends, besides on genotype, on growing conditions, especially temperature, as well as on maturity stage. Lower starch contents can be the result of either sub- or supra-optimal temperatures during grain filling (Anker-Nilssen *et al*., 2006). High temperatures during grain filling can impede starch accumulation in combination with an increased growth rate and a

reduced grain-filling duration (Muchow, 1990). High temperatures may also impair starch synthesis, with less starch per endosperm and smaller starch granules or change the composition (amylose: amylopectin) (Tester *et al*., 1991; 1995). Lower temperatures may result in less starch accumulation and lower starch contents because of a slower and limited grain filling, despite the advantage of longer growth duration (Muchow, 1990; Wilson *et al*., 1995).

Different maize genotypes show differences in earliness and rate of maturation (Tollenaar, 1989; Rebourg *et al*., 2003) and, therefore, in their response to growing temperatures. This makes it difficult to understand how genotype and growing conditions interact on starch accumulation and finally on starch degradation (Ettle and Schwarz, 2003). Dry matter (DM) content is a good descriptor of maturity (Jensen *et al*., 2005) and is an important tool to rank maize genotypes based on their maturity (Schwab *et al*., 2003; Marton *et al*., 2007). However, maize genotypes can differ in their nutritive value, even at the same dry matter content (Hetta *et al*., 2012; Jensen *et al*., 2005).

The present study aimed to understand how growing conditions, maturity stage and genotype interact to influence *in vitro* starch degradation of maize kernels in rumen fluid.

2. Materials and methods

2.1. Maize kernel samples

Maize kernel samples were collected from two glasshouse experiments conducted at UNIFARM, Wageningen, The Netherlands. Each experiment included six genotypes and three day/night temperature regimes during grain filling (reproductive phase) with three replications. The genotypes were selected on the basis of variation in amylose content and type of endosperm, i.e. vitreousness. Four genotypes (dent, flint, high amylose and waxy) were used in both experiments. In Exp. 1, also the normal counter parts of the high amylose and waxy genotypes were used while in Exp. 2 also non-vitreous and vitreous endosperm (rumen escaping) types were used. The high amylose counterpart and the waxy counterpart had both similar amylose contents but were from different inbred lines. An overview of the

Table 1

Maize genotypes and temperature used in the two greenhouse experiments.

Genotype	Maturity type	Amylose content (%)	Exp. 1	Exp. 2
Homozygous dent	FAO300[2]	20-30	X	
Homozygous flint	FAO170	20-30	X	X
High amylose	FAO240	>50	X	X
High amylose CP[1]	FAO240	20-30	X	
Waxy CP	FAO240	20-30	X	
Waxy	FAO240	0	X	X
Non-vitreous				X
Vitreous (rumen escaping)				X
Temperature (°C, day/night)				
Low			18/12	22/12
Intermediate			24/18	27/17
High			30/24	32/22

[1]CP: Counterpart; [2]FAO: Food and Agriculture Organization.

types of maize used in each experiment is given in Table 1. The average day/night temperature treatments after pollination were 18/12 °C, 24/18 °C and 30/24 °C with 12 h light and 12 h dark in Exp. 1, and 22/12 °C, 27/17 °C and 32/22 °C with 15 h light and 9 h dark in Exp. 2. Cobs were harvested; husk leaves removed and kernels were manually removed from the cobs on the same day using a sharp knife. The kernels were removed from the middle part of the cob to maintain uniformity. The samples were collected when the starch content in the kernels was between 368 and 633 g/kg organic matter (OM) (350-500 °Cd, 24-43 d after pollination) and between 558 and 674 g/kg OM (500-700 °Cd, 34-63 d after pollination) in Exp. 1. In Exp. 2, the kernels were harvested at starch contents of 401-618 g/kg OM (300-400 °Cd, 22-29 d after pollination) and of 537-695 g/kg OM (550-750 °Cd, 37-55 d after pollination). These samples were collected from one plant per replication in Exp. 1 and from two plants per replication in Exp. 2. The different treatment combinations are illustrated in Table 1. The weight of 60 randomly chosen kernels from each cob was recorded and the kernels were subsequently dried at 70 °C for 48 h in a forced

ventilation oven, reweighed and ground to pass a 1 mm sieve, using a centrifugal mill (Retsch ZM 100, Haan, Germany), and stored at room temperature.

2.2. Chemical analysis

Dry matter content after storage of dried kernels was determined gravimetrically by drying for 4 h at 103 °C (ISO 6496), and ash was determined by incineration for 3 h at 550 °C (ISO 5984). Starch content was determined using the amyloglucosidase method described by Keppler and Decker (1970). The amount of starch per kernel was calculated by multiplying the starch concentration of the ground sample per unit dry matter with individual kernel dry matter weight.

2.3. In vitro *rumen fermentation and starch degradation*

Ground whole kernels were incubated in buffered rumen fluid, using the gas production technique as described by Cone *et al*. (1996). Rumen fluid was collected 2 h after the morning feeding from two rumen fistulated lactating cows, fed a mixture of grass (2/3) and maize silage (1/3) and concentrate to requirements. Samples of 0.5 g of substrate were incubated in duplicate in 60 ml buffered rumen fluid in 250 ml bottles placed in a shaking water bath, maintained at 39 °C, using the procedure described by Cone *et al*. (1996) Gas production was recorded for 72 h. Results were corrected for blank gas production, i.e. gas production in buffered rumen fluid without substrate. Within each run, consisting of 40 samples in duplicate, a pure starch (control) and two standard maize samples (normal and gelatinized) accompanied the test maize samples to allow standardisation. T-tests showed no significant differences in gas production between batches for the control ($P>0.51$) and standard samples ($P>0.23$ and 0.40 for normal and gelatinized starch, respectively). Batches were, therefore, not corrected for external standard differences.

Based on the volume of gas production at 6, 12 and 20 h and the starch content of the samples, starch degradation was calculated for 6, 12 and 20 h of incubation in rumen fluid, respectively, using the equation of Chai *et al*. (2004):

Starch degradation at time t (g/kg OM) = -191.6(±14.6) + 0.303(±0.025) × Starch content (g/kg OM) + 1.648(±0.053) × Gas produced at t (ml/g OM)

and the following equation to calculate *in vitro* rumen starch degradation per gram of starch:

Starch degradation (g/kg starch) = [Starch degradation (g/kg OM) / Starch content (g/kg OM)] × 1000

2.4. Statistical analysis

The following model and the GLM procedure in SAS (2002) was used to determine genotype and temperature effects on starch content, amount of starch per kernel, whole kernel degradation and estimated starch degradation (after 6, 12 or 20 h incubation):

$$Y_{ijk} = \mu + G_i + T_j + R_k + G_i \times T_j + G_i \times R_k + T_j \times R_k + \varepsilon_{ijk}$$

where

Y_{ijk} = Starch contents, amount of starch per kernel, whole kernel degradation or starch degradation at 6, 12 and 20 h;
μ = overall mean;
G_i = genotype;
T_j = temperature;
R_k = replication;
ε_{ijk} = general error term.

3. Results

3.1. Starch contents

Starch content was significantly influenced by genotype ($P<0.0001$) and temperature ($P<0.0001$) at two different maturity stages in Exp. 1 (Tables 2 and 3) and Exp. 2 (Tables 4 and 5). Starch contents showed a sigmoidal relationship when plotted against thermal time (Fig. 1). The highest starch contents were obtained for

the waxy and the counter parts of amylose and waxy at both maturity stages in Exp. 1, and non-vitreous at the earlier and vitreous (rumen escaping) at the later maturity stage in Exp. 2. The intermediate temperatures (24/18 °C in Exp. 1 and 27/17 °C in Exp. 2) gave the highest starch content in both experiments, whereas the lowest contents were observed at the low temperature regime (18/12 °C) in Exp. 1 (Tables 2 and 3), and at the high temperature regime (32/22 °C) in Exp. 2 (Tables 4 and 5). Starch content was also influenced by the maturity stage (thermal time). The later maturity stages gave higher starch contents than the earlier stages. The variance in starch content between the genotypes and temperature regimes at earlier maturity stages was larger and reduced with advancing maturity (Fig. 1) in both experiments.

3.2. Amount of starch per kernel

The amount of starch per kernel was significantly influenced by genotype ($P<0.0001$) and temperature regime ($P<0.0001$) at the two different maturity stages in each experiment (Tables 2-5) and showed a similar trend as the starch content (g/kg), when plotted against thermal time (Fig. 1). The highest amount of starch per kernel was observed in the waxy, counterparts of waxy, high amylose and dent in Exp. 1 (Tables 2 and 3), and waxy at the earlier and vitreous at the later maturity stages in Exp. 2 (Tables 4 and 5). The highest amount of starch per kernel was observed at the intermediate temperature (24/18 and 27/17 °C in Exps 1 and 2, respectively) at all maturity stages in both experiments, whereas the lowest amount of starch was observed at the low temperature (18/12 °C) in Exp. 1, and the high temperature (32/22 °C) in Exp. 2 (Tables 2-5). Maturity stage also significantly influenced the amount of starch per kernel in the same way as it influenced starch content: the later maturity stages gave higher amounts of starch per kernel than the earlier stages (Fig. 1).

Table 2
Starch content, amount of starch per maize kernel, whole kernel gas production and calculated starch degradation at 6, 12 and 20 h as influenced by maize genotype and growing temperature during grain filling when harvested at starch contents of 368-633 g/kg OM in Exp. 1.

Temperature (T, °C) day/night	Genotype (G)	Starch contents, g/kg OM		Starch/kernel, mg		Rumen degradation					
						Whole kernel, ml/g OM		Starch, g/kg starch			
		Mean	SEM	Mean	SEM	72 h	SEM	6 h	12 h	20 h	Pooled SEM
18/12	Dent	531	3.4	80	6.0	298	2.4	380	689	782	9.4
	Flint	508	2.9	67	3.5	273	3.9	334	626	725	10.9
	High amylose	368	13.8	45	3.5	227	3.2	290	571	667	12.1
	High amylose CP	510	24.3	79	6.0	287	12.4	339	702	767	14.0
	Waxy CP	521	4.5	75	11.5	296	4.7	370	713	789	10.1
	Waxy	529	4.8	83	2.5	305	3.0	394	720	795	7.7
24/18	Dent	578	7.8	100	2.0	335	6.5	418	735	832	8.8
	Flint	551	4.4	89	3.0	309	0.4	371	685	790	6.2
	High amylose	478	12.0	80	11.0	275	5.4	339	632	733	4.7
	High amylose CP	592	49.2	107	5.5	326	6.9	416	734	807	9.7
	Waxy CP	581	18.8	112	3.0	340	9.3	424	743	831	7.8
	Waxy	633	0.1	114	7.0	366	2.2	441	748	827	6.1
30/24	Dent	558	6.3	86	4.0	316	4.6	385	706	804	7.8
	Flint	515	10.3	75	0.5	282	1.7	325	643	744	6.5
	High amylose	455	6.0	58	2.5	252	0.1	284	602	691	8.0
	High amylose CP	553	0.5	84	2.5	319	3.0	393	716	816	8.3
	Waxy CP	553	5.8	92	2.0	320	6.1	402	726	813	9.8
	Waxy	547	2.0	91	3.0	320	0.5	404	734	809	7.9
P-value											
T		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	18/12	495^{c}		71^{c}		281^{c}		351^{c}	670^{c}	754^{c}	
	24/18	569^{a}		100^{a}		325^{a}		401^{a}	713^{a}	803^{a}	
	30/24	530^{b}		81^{b}		301^{b}		365^{b}	688^{b}	779^{b}	
G		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	Dent	556^{a}		89^{a}		317^{b}		394^{bc}	710^{c}	806^{ab}	
	Flint	525^{b}		77^{b}		288^{c}		343^{d}	651^{d}	753^{c}	
	High amylose	434^{c}		61^{c}		251^{d}		304^{e}	602^{e}	697^{d}	
	High amylose CP	552^{a}		90^{a}		311^{b}		383^{c}	717b^{c}	796^{b}	
	Waxy CP	552^{a}		92^{a}		319^{b}		398^{ab}	727^{ab}	811^{a}	
	Waxy	570^{a}		96^{a}		330^{a}		413^{a}	734^{a}	810^{ab}	
T × G		0.115		0.795		0.068		0.083	0.415	0.147	

Means followed by different letters are significantly (P<0.05) different.
OM: Organic matter; CP: Counterpart.
Starch degradation measurements were conducted in two runs. Gas production values of control and standard samples did not differ (P >0.05) between the runs.

Table 3
Starch content, amount of starch per maize kernel, whole kernel gas production and calculated starch degradation at 6, 12 and 20 h as influenced by maize genotype and growing temperature during grain filling when harvested at starch contents of 558-674 g/kg of OM in Exp. 1.

Temperature (T, °C) day/night	Genotype (G)	Starch contents, g/kg OM		Starch/kernel, mg		Rumen degradation					
						Whole kernel, ml/g OM		Starch, g/kg starch			
		Mean	SEM	Mean	SEM	72 h	SEM	6 h	12 h	20 h	Pooled SEM
18/12	Dent	621	1.0	150	7.5	285	1.0	243	568	649	5.9
	Flint	576	6.4	108	2.5	273	4.5	200	534	611	9.2
	High amylose	558	9.5	101	3.0	248	0.5	133	461	554	8.1
	High amylose CP	629	3.3	134	11.5	289	3.0	232	575	644	7.4
	Waxy CP	635	3.2	146	9.0	300	0.2	248	590	662	14.4
	Waxy	632	7.3	141	8.5	312	7.3	255	603	669	7.5
24/18	Dent	640	8.2	194	2.5	303	1.8	272	597	698	4.7
	Flint	622	7.0	181	6.5	302	2.3	248	572	672	8.4
	High amylose	596	10.8	158	5.5	285	2.2	174	499	600	8.1
	High amylose CP	665	4.2	176	5.0	338	15.3	299	620	723	6.8
	Waxy CP	674	7.2	185	5.0	330	5.8	301	629	730	8.3
	Waxy	670	2.8	187	4.0	328	1.0	305	619	734	5.2
30/24	Dent	645	2.4	168	3.5	294	2.4	264	577	669	3.0
	Flint	591	5.8	135	6.5	274	6.4	209	531	632	5.2
	High amylose	586	3.6	123	11.5	266	1.2	142	484	565	6.9
	High amylose CP	657	6.1	159	3.0	316	2.3	276	609	707	1.9
	Waxy CP	658	5.7	170	2.5	315	1.0	279	608	707	9.0
	Waxy	658	0.6	173	1.5	321	3.2	300	615	712	4.9
P-value											
T		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	18/12	609^{c}		130^{c}		285^{c}		219^{c}	555^{c}	632^{c}	
	24/18	645^{a}		180^{a}		314^{a}		266^{a}	589^{a}	693^{a}	
	30/24	632^{b}		154^{b}		298^{b}		245^{b}	571^{b}	665^{b}	
G		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	Dent	635^{b}		170^{a}		294^{b}		260^{c}	581^{c}	672^{c}	
	Flint	596^{c}		141^{c}		283^{c}		219^{d}	545^{d}	638^{d}	
	High amylose	580^{d}		127^{d}		266^{d}		150^{e}	481^{e}	573^{e}	
	High amylose CP	650^{a}		156^{b}		314^{a}		269^{bc}	601^{b}	691^{b}	
	Waxy CP	656^{a}		167^{ab}		315^{a}		276^{ab}	609^{ab}	700^{ab}	
	Waxy	653^{a}		167^{ab}		320^{a}		287^{a}	612^{a}	705^{a}	
T × G		0.247		0.325		0.197		0.083	0.130	0.088	

Means followed by different letters are significantly (P<0.05) different.
OM: Organic matter; CP: Counterpart.
Starch degradation measurements were conducted in two runs. Gas production values of control and standard samples did not differ (P >0.05) between the runs.

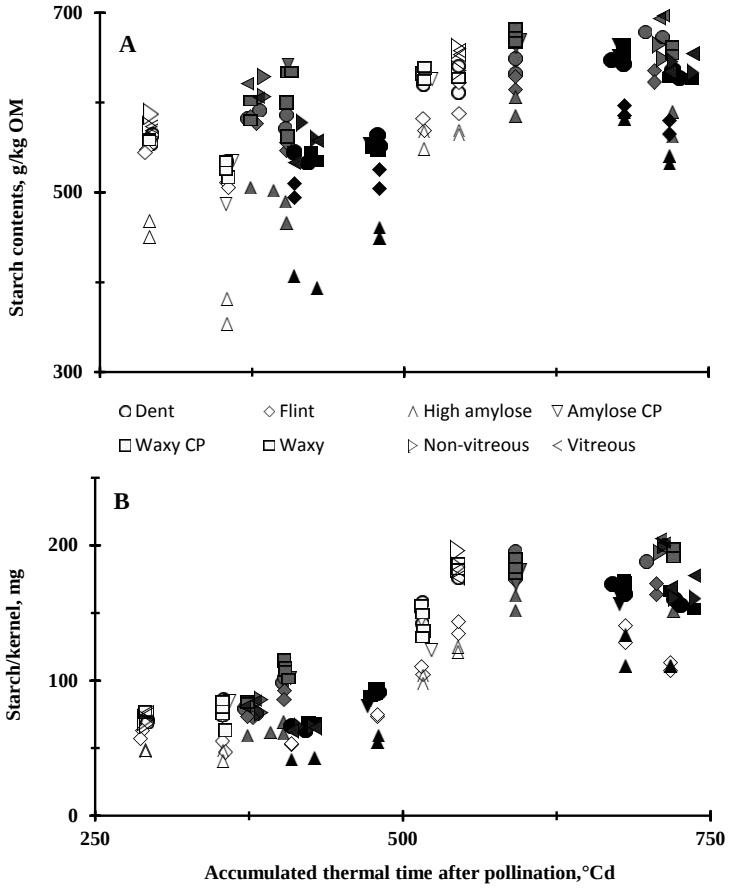

Fig. 1. Relationship between starch content (panel A) or amount of starch per kernel (panel B) and accumulated growth thermal time during grain filling. Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 ºC in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 ºC in Exp. 2) and high (30/24 in Exp. 1 and 32/22 ºC in Exp. 2) temperature treatments during grain filling, respectively.

3.3. Starch degradation in rumen fluid

The 72 h gas production (ml/g OM) of the ground whole kernels in rumen fluid differed significantly among genotypes ($P<0.0001$) and temperature treatments ($P<0.0001$-0.003) at all maturity stages (Tables 2-5). High amylose showed the lowest gas production at all temperature treatments and maturity stages in both experiments. The highest gas production was observed for waxy in Exp. 1 (Tables 2

and 3), and non-vitreous and waxy in Exp. 2 (Tables 4 and 5). The higher rumen *in vitro* starch degradation values were observed for the counterpart of waxy and for waxy itself at lower (368-633 g/kg OM) starch contents (Table 2) than for the other genotypes in the study. At higher (558-674 g/kg of OM) starch contents, waxy and its counterpart gave higher rumen starch degradation values (Exp. 1, Table 3) than other genotypes under investigation. The highest rumen starch degradation values were observed for non-vitreous and waxy maize genotypes in Exp. 2 at both levels of starch contents (Tables 4 and 5). The highest values of gas production were observed for kernels grown at the intermediate temperatures (24/18 and 27/17 °C) in both experiments. The lower temperature (18/12 °C) in Exp. 1 (Tables 2 and 3) and the higher temperature (32/22 °C) in Exp. 2 (Tables 4 and 5) resulted in the lowest gas productions. The calculated starch degradation, using the equation of Chai *et al*. (2004) for 6, 12 and 20 h of incubation in rumen fluid was significantly influenced by genotype and growth temperature at all maturity stages in both experiments (Tables 2-5). The highest ruminal starch degradation was observed for the counterpart of waxy (811 g/kg starch after 20 h incubation, Table 2) and waxy itself (705 g/kg starch after 20 h incubation, Table 3) in Exp. 1. The non-vitreous type gave the highest estimates for starch degradation after 20 h of incubation (773 g/kg starch and 688 g/kg starch) in Exp. 2. This was found consistently for all starch contents (Fig. 2) and amount of starch per kernel (Fig. 3) at all maturity stages. The relationship between rumen starch degradation of the genotypes against their starch contents (Fig. 2) and the amount of starch per kernel (Fig. 3) showed that all the genotypes showed more or less the same trend at two comparisons except for the vitreous genotype. The latter gave the lowest values for estimated gas production and starch degradation (Exp. 2) even at relatively high starch contents (Fig. 2) and large amounts of starch per kernel (Fig. 3). The high amylose genotype had the lowest starch content (Fig. 1), gas production and rumen starch degradation in Exp. 1 (Figs 2 and 3), whereas in Exp. 2, vitreous showed the lowest starch degradation values (Tables 4 and 5). Maturity stage strongly influenced the *in vitro* rumen starch degradation. Rumen starch degradation declined when maturation progressed, even

with higher starch contents (Tables 2-5, Fig. 2) or larger amounts of starch per kernel (Fig. 3).

Table 4

Starch content, amount of starch per maize kernel, whole kernel gas production and calculated starch degradation at 6, 12 and 20 h as influenced by maize genotype and growing temperature during grain filling when harvested at starch contents of 401-618 g/kg of OM in Exp. 2.

Temperature (T, °C) day/night	Genotype (G)	Starch contents, g/kg OM		Starch/kernel, mg		Rumen degradation					
						Whole kernel, ml/g OM		Starch, g/kg starch			
		Mean	SEM	Mean	SEM	72 h	SEM	6 h	12 h	20 h	Pooled SEM
22/12	Dent	559	5.0	70	0.5	298	2.9	375	682	741	6.3
	Flint	546	1.7	60	3.0	284	0.6	333	669	713	5.6
	High amylose	459	8.9	49	0.5	255	1.4	277	580	668	6.7
	Waxy	558	0.6	77	1.0	308	2.9	374	720	773	7.1
	Non-vitreous	580	7.3	70	1.5	320	3.1	390	722	774	7.0
	Vitreous	571	2.2	76	1.0	276	0.5	272	555	640	5.9
27/17	Dent	587	4.5	77	2.0	317	0.7	399	728	767	5.4
	Flint	580	3.9	73	0	304	1.7	354	693	750	6.6
	High amylose	504	1.7	61	1.5	264	2.9	302	619	680	9.8
	Waxy	591	11.1	84	1.0	321	0.1	411	733	782	9.6
	Non-vitreous	618	11.2	81	5.0	335	0.4	412	740	783	5.6
	Vitreous	613	7.8	83	2.5	286	2.4	292	590	669	13.4
32/22	Dent	539	5.6	65	1.5	286	3.9	360	657	727	4.7
	Flint	502	7.8	54	0.5	274	12.6	314	630	694	6.2
	High amylose	401	6.6	43	0.5	226	0.8	275	591	642	11.7
	Waxy	540	5.0	69	0	279	26.5	380	702	759	5.9
	Non-vitreous	569	8.9	66	0	311	1.0	389	707	760	2.6
	Vitreous	545	12.2	64	1.0	280	28.6	254	564	591	11.7
P-value											
T		<.0001		<.0001		0.003		<.0001	<.0001	<.0001	
	22/12	546[b]		67[b]		290[b]		337[b]	655[b]	718[b]	
	27/17	582[a]		76[a]		304[a]		362[a]	684[a]	739[a]	
	32/22	515[c]		60[c]		276[c]		339[c]	642[c]	696[c]	
G		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	Dent	562[b]		70[c]		300[b]		378[b]	689[b]	745[b]	
	Flint	543[c]		62[d]		287[bc]		333[c]	664[c]	719[c]	
	High amylose	455[d]		51[e]		249[d]		285[d]	597[d]	663[d]	
	Waxy	563[b]		77[a]		303[ab]		388[a]	718[a]	772[a]	
	Non-vitreous	589[a]		72[bc]		322[a]		397[a]	723[a]	773[a]	
	Vitreous	576[a]		74[ab]		281[c]		273[e]	570[e]	634[e]	
T × G		0.145		0.260		0.842		0.480	0.094	0.068	

Means followed by different letters are significantly (P<0.05) different.
OM: Organic matter; CP: Counterpart.
Starch degradation measurements were conducted in two runs. Gas production values of control and standard samples did not differ (P >0.05) between the runs.

Table 5
Starch content, amount of starch per maize kernel, whole kernel gas production and calculated starch degradation at 6, 12 and 20 h as influenced by maize genotype and growing temperature during grain filling when harvested at starch contents of 537-695 g/kg of OM in Exp. 2.

Temperature (T, °C) day/night	Genotype (G)	Starch contents, g/kg OM		Starch/kernel, mg		Rumen degradation					
						Whole kernel, ml/g OM		Starch, g/kg starch			
		Mean	SEM	Mean	SEM	72 h	SEM	6 h	12 h	20 h	Pooled SEM
22/12	Dent	626	14.8	181	0.5	285	2.4	258	597	663	5.5
	Flint	605	17.5	139	3.0	271	6.2	239	563	649	4.6
	High amylose	567	2.2	123	0.5	250	1.75	163	499	586	7.3
	Waxy	627	0.1	186	1.0	288	3.1	299	630	684	4.0
	Non-vitreous	648	12.0	187	1.5	300	6.1	291	633	689	3.4
	Vitreous	655	2.1	187	1.0	274	3.0	139	453	537	8.2
27/17	Dent	676	2.7	194	2.0	302	11.6	315	616	675	9.2
	Flint	629	6.0	168	0	289	1.9	267	579	665	7.6
	High amylose	576	13.7	159	1.5	258	2.1	195	513	605	5.7
	Waxy	658	4.5	195	1.0	299	4.8	328	646	705	3.5
	Non-vitreous	656	8.1	197	5.0	310	5.4	332	648	705	6.1
	Vitreous	695	1.4	204	2.5	289	3.0	174	493	575	3.6
32/22	Dent	632	4.7	154	1.5	265	9.4	239	574	632	9.9
	Flint	573	7.5	120	0.5	258	0.7	228	546	608	5.1
	High amylose	537	4.2	106	0.5	243	0.4	146	484	569	6.5
	Waxy	627	0.7	159	0	275	1.7	273	611	665	7.0
	Non-vitreous	639	6.5	161	0	269	6.7	279	616	671	8.2
	Vitreous	645	10.1	169	1.0	279	8.0	127	418	502	7.1
P-value											
T		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	22/12	621^{b}		167^{b}		278^{b}		232^{b}	563^{b}	635^{b}	
	27/17	648^{a}		186^{a}		291^{a}		268^{a}	583^{a}	655^{a}	
	32/22	608^{c}		145^{c}		265^{c}		215^{c}	542^{c}	608^{c}	
G		<.0001		<.0001		<.0001		<.0001	<.0001	<.0001	
	Dent	644^{b}		176^{b}		284^{abc}		271^{b}	596^{b}	657^{b}	
	Flint	602^{c}		142^{c}		273^{c}		245^{c}	563^{c}	641^{c}	
	High amylose	560^{d}		129^{d}		251^{d}		168^{d}	498^{d}	587^{d}	
	Waxy	637^{b}		180^{ab}		288^{ab}		300^{a}	629^{a}	685^{a}	
	Non-vitreous	647^{b}		181^{ab}		293^{a}		301^{a}	633^{a}	688^{a}	
	Vitreous	665^{a}		186^{a}		281^{bc}		147^{e}	454^{e}	538^{e}	
T × G		0.270		0.372		0.366		0.213	0.059	0.152	

Means followed by different letters are significantly (P<0.05) different.
OM: Organic matter; CP: Counterpart.
Starch degradation measurements were conducted in two runs. Gas production values of control and standard samples did not differ (P >0.05) between the runs.

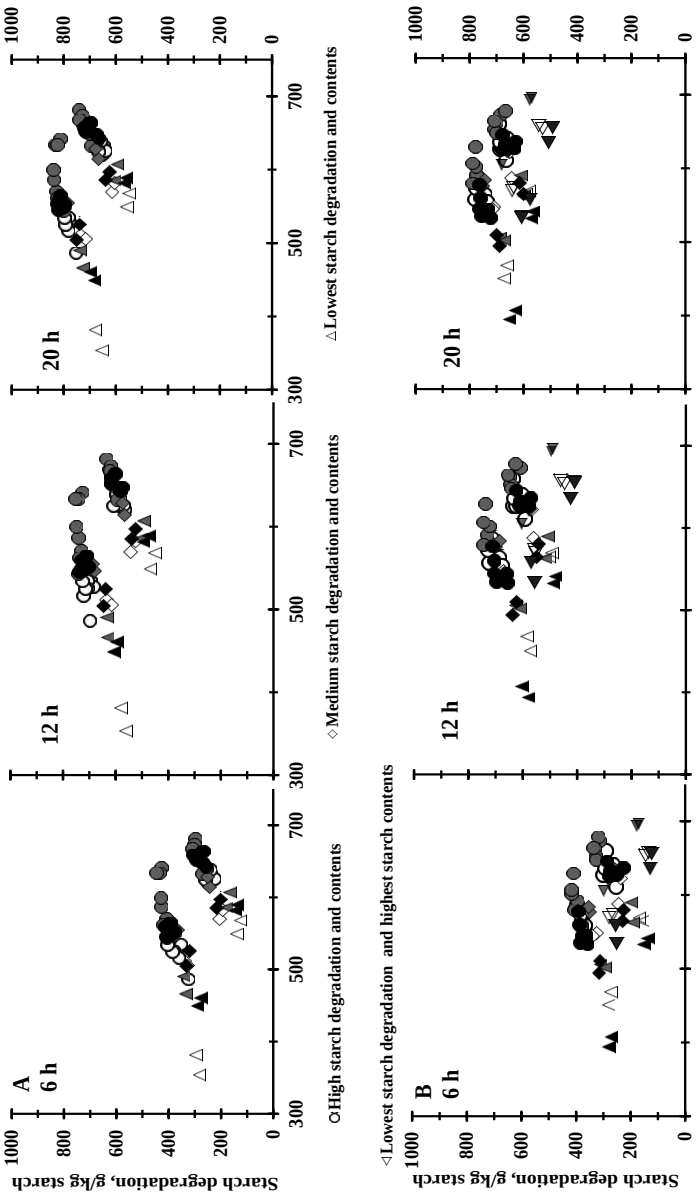

Fig. 2. Relationship between rumen starch degradation (mg/g starch) of 6, 12 and 20 h incubated maize kernels against their starch contents (g/kg) at three different growth temperature treatments during grain filling in Exp. 1 (panel A) or in Exp. 2 (panel B). Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 °C in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 °C in Exp. 2) and high (30/24 in Exp. 1 and 32/22 °C in Exp. 2) temperature treatments during grain filling, respectively.

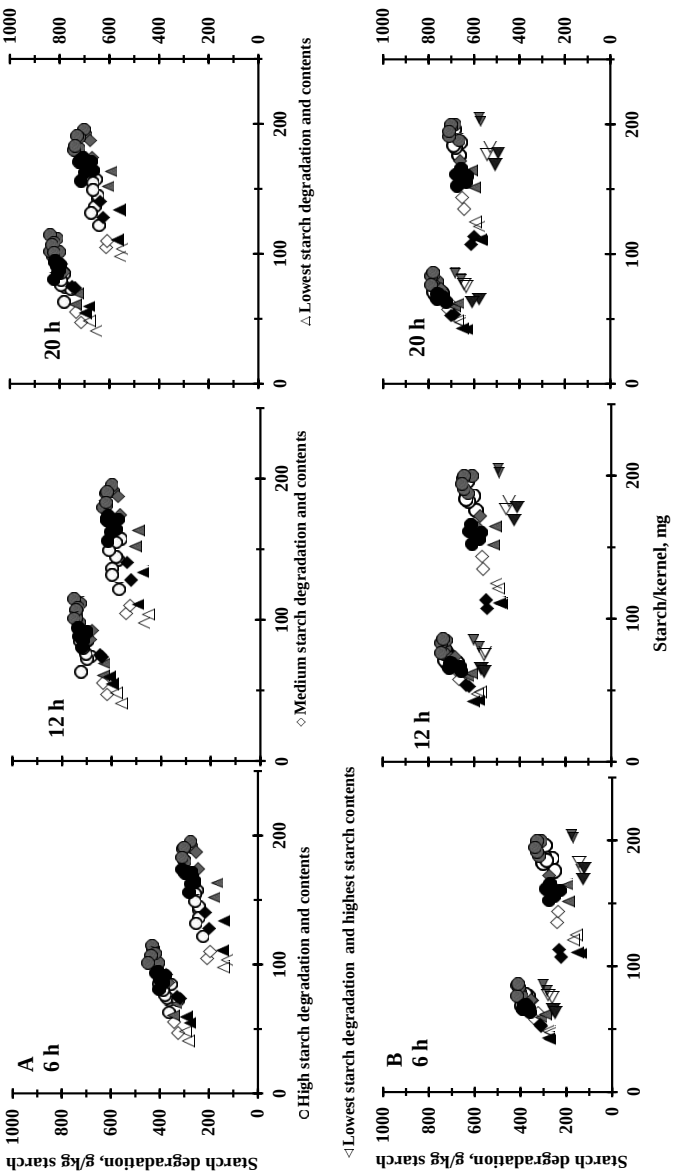

Fig. 3. Relationship between rumen starch degradation (mg/g starch) of 6, 12 and 20 h incubated maize kernels against their amount of starch per kernel (mg) at three different growth temperature treatments during grain filling in Exp. 1 (panel A) or in Exp. 2 (panel B). Lower and upper data shows ruminal starch degradation at higher starch contents (latest maturity stage) and lower starch contents (earlier maturity stage) respectively. Open, grey and black markers are for low (18/12 in Exp. 1 and 22/12 ºC in Exp. 2), intermediate (24/18 in Exp. 1 and 27/17 ºC in Exp. 2) and high (30/24 in Exp. 1 and 32/22 ºC in Exp. 2) temperature treatments during grain filling, respectively.

4. Discussion

Rumen *in vitro* starch degradation (calculated) of maize kernels at all incubation times was significantly influenced by starch structure (i.e. amylose or amylopectin), starch composition (i.e. amylose: amylopectin), starch content and type of endosperm (i.e. vitreousness). These variables strongly depend on growing conditions, genotype and maturity stage. Within a certain genotype high ruminal starch degradability can be either associated with low starch content or with a high starch content, depending on starch structure (i.e. amylose or amylopectin) and composition (i.e. amylose: amylopectin) (Kotarski *et al*., 1992; Opatpatanakit *et al*., 1994; Philippeau *et al*., 1998). Starch structure and composition are genotype-specific. Starch accumulation also depends on growing conditions, and maturity stage determines starch contents, hence influencing rumen starch degradation. In our experiments we did not find significant interactions between temperature and genotype.

We found increasing starch degradation in rumen fluid with increasing starch contents at all incubation times up to a certain maturity stage, in all maize genotypes (high amylose, non-vitreous, waxy). The low rumen starch degradation in the high amylose genotype (Exp. 1) is associated with its high amylose content, as rumen starch degradation is negatively correlated with amylose content (Li *et al*., 2001; 2004; Anker-Nilssen *et al*., 2006; Stevnebø *et al*., 2006). This is likely due to the fact that only amylose can be in a crystalline form (Sadeghi and Shawrang, 2006). The crystalline starch structure is possibly the most important factor determining its degradability (Wolters and Cone, 1992; Zhang and Oates, 1999) as crystallinity reduces the accessibility for degrading enzymes (Vasanthan and Bhatty, 1996). This also explains why the waxy genotype showed higher rumen starch degradation than the high amylose genotype in our experiments, as the waxy genotype has no amylose. Lichtenwalner *et al*. (1978) showed increased degradability with increased amylopectin or decreased amylose. This was also confirmed *in vitro* (Kotarski *et al*., 1992; Opatpatanakit *et al*., 1994; Philippeau *et al*., 1998) and *in vivo* (Philippeau *et al*., 1998) by other authors.

The higher rumen starch degradation of dent (*Zea mays indentata*) than flint (*Zea mays indurata*) in our study could be the result of a different type of endosperm with lower vitreousness in dent (Lichtenwalner *et al*., 1978; Hibberd *et al*., 1982). Vitreous starch granules are highly dense and yellowish in colour, and the molecules are more close, making them lesser degradable than dent. Vitreousness increases with maturity (Pereira *et al*., 2004). The more vitreous the starch and the denser the network, the less accessible the granules are for enzymatic degradation (Cui and Oates, 1999; Vesterinen *et al*., 2002; Svihus *et al*., 2005), also reducing the starch degradation in the rumen (Holm *et al*., 1983; Vasanthan and Bhatty, 1996). Therefore, we can conclude that non-vitreous starch structure and higher amylopectin content, along with higher starch content in any maize genotype at the same maturity stage results in a higher degradability.

Starch accumulation showed an optimum growth temperature at 24/18 °C (Exp. 1) and 27/17 °C (Exp. 2) during grain filling. The two temperature regimes below or above these levels, negatively influenced starch accumulation. Consequently, unfavourable growth temperatures significantly reduced the rumen starch degradation at the same maturity stage. The lower starch degradation at lower temperatures (18/12 °C in Exp. 1) could be due to less starch accumulation, as the mean temperature (15 °C) was below the optimum of 19 °C. Growth temperatures lower than 16 °C result in an inefficient use of intercepted radiation, reducing crop growth rate, and prolonging the grain filling period (Wilson *et al*., 1995), causing less starch accumulation and lower starch contents (Muchow, 1990; Wilson *et al*., 1995). In contrast, lower rumen starch degradation of maize, grown at temperatures above that optimum (Exp. 2), is caused by impaired starch synthesis (Jenner, 1994) resulting in less starch per kernel (Tester *et al*., 1995). Elevated temperatures also block the kernel development (Commuri and Jones, 2001) due to a reduced grain filling duration (Bhullar and Jenner, 1986) resulting in less starch accumulation and lower starch contents at grain maturity Moreover supra-optimal temperatures may result in a small increase in amylose content, i.e. less degradable starch (Anker-Nilssen *et al*., 2006). Higher growth temperature can also reduce accessibility of glucan chain ends for the action of amyloglucosidase (Morrison *et al*., 1986;

Commuri and Jones, 2001) by reducing surface area. It also results in complexes like starch–protein matrixes of the endosperm (Anker-Nilssen *et al*., 2006) and amylose-fatty acids complexes (Tester *et al*., 1991; Tester, 1997). These complexes reduce the rate of enzymatic degradation of starch (Cui and Oates, 1999; Crowe *et al*., 2000) and limit ruminal starch degradation (McAllister *et al*., 1993).

Starch content and rumen starch degradation depended on the maturity stage at which maize kernels were harvested. Starch degradation of the maize kernels of different genotypes grown under different conditions, showed similar trends at all incubation times in rumen fluid, independent of the maturity stage. The latest maturity stage showed the highest starch contents (Hetta *et al*., 2012), but ruminal starch degradation was lower as compared with earlier harvested samples, which were lower in starch contents. The lower starch degradation at later maturity stages could be because of increased virtuousness of the maize kernels (Tolera *et al*., 1998; Tolera and Sundstøl, 1999; Ettle *et al*., 2001). This suggests that the higher the degree of vitreousness, the lower the ruminal starch degradation will be and *vice versa*. This was confirmed in the present study. As there are changes in the chemical composition of maize kernels, up to a certain maturity stage (Struik, 1983). That is why we found a prominent effect of maturity on gas production and rumen starch degradation up to a certain maturity stage.

5. Conclusion

The *in vitro* ruminal starch degradation of maize kernels was significantly influenced by genotype and growth temperature during grain filling. The genotypes with a lower amylose contents and higher starch contents with no or less vitreousness degraded to a higher extent at all growth temperatures, maturity stages and incubation times, whereas growth temperature affected rumen starch degradation negatively at lower and higher than optimum growth temperature, due to its effect on starch accumulation rate and content. Less starch was degraded in rumen fluid under unfavourable lower (due to slower accumulation rate) and higher growth temperatures (faster accumulation rate but reduced grain filling duration) at the same maturity stage. Maturity stage also played a significant role and higher

rumen starch degradation values were observed at earlier (lower starch contents), rather than at a later maturity stage (higher starch contents) regardless of genotypes and growth temperature. This suggests that a higher rumen starch degradation of maize kernels is maturity specific. At early or late maturity stages rumen starch degradation is relatively low.

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Oven-drying reduces ruminal starch degradation in maize kernels

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Chapter 4

Submitted to Animal Feed Science and Technology

ABSTRACT

The degradation of starch largely determines the feeding value of maize (*Zea mays* L.) for dairy cows. Normally, maize kernels are dried and ground before chemical analysis and determining degradation characteristics, whereas cows eat and digest fresh material. Drying the moist maize kernels (consisting mainly of starch) at high temperatures can influence their physical properties and thus their degradation dynamics in the rumen. We compared the *in vitro* degradability of dried maize kernels with that of fresh kernels after incubation in rumen fluid. Maize kernels were obtained from genotypes diverse in starch structure, composition and type of endosperm. These genotypes were grown in greenhouses at different temperatures during starch accumulation, and harvested at different maturity stages, in two experiments. Starch content was assessed using the amyloglucosidase method. Fermentation in rumen fluid was measured using an *in vitro* gas production technique. Starch degradation of the kernels was calculated after 6, 12 and 20 h of incubation in rumen fluid. Oven-drying significantly ($P<0.0001$) influenced the *in vitro* degradation of starch in maize kernels at the different incubation times, with more starch being degraded in the fresh than in the oven-dried maize kernels, although the differences were small (11-15%). There was a consistent and highly significant ($P<0.009$ to 0.0002) interaction between oven-drying and genotype, with the high-amylose genotype showing larger effects of oven-drying than the other genotypes. The vitreous genotype showed lower starch degradation than the non-vitreous type. At earlier maturity stages, the difference between oven-dried and fresh kernels was larger than at later maturity stages. The temperature during grain filling significantly ($P<0.0001$) affected starch degradation but did not affect the difference between fresh and oven-dried samples. Oven-drying significantly reduced the *in vitro* rumen starch degradation of maize kernels regardless of growing conditions, genotype and maturity stage, but its effect depends on genotype and maturity.

Keywords: Maize (*Zea mays* L.) kernels, Oven-drying, Starch degradation, Feeding value, Ruminants.

1. Introduction

Maize (*Zea mays* L.) is a major component in the ration of dairy cows in many parts of the world. The feeding value of maize for ruminants largely depends on the starch content and its degradation characteristics (Canizares *et al*., 2011; Theurer, 1986). Maize starch degradability is mainly affected by its physical characteristics and can be altered through processing (Cone and Vlot, 1990; Yang *et al.*, 2001) with post-harvest processing normally increasing the degradability of starch (Andrae *et al*., 2001; Yang *et al*., 2001). However, drying moist maize kernels (starch) at high temperatures can also cause changes to its physical properties by rearranging the amylose molecules (retro-gradation) and, thereby, decreasing its degradability (Rooney and Pflugfelder, 1986). Moreover, Maillard reactions and enzymatic browning may also occur during drying (Maillard, 1912) and can play an important role in the feeding quality of maize. The Maillard reaction is a non-enzymatic browning reaction between carbonyl and amino compounds, which occurs in foods containing protein and carbohydrates (Ong and Law, 2010).

Normally samples of feeds and feed ingredients are oven-dried before chemical analysis and determination of their degradation characteristics, either *in vitro* or *in sacco* (Deinum and Maassen, 1994). As the starch is not consumed by dairy cows in the form of dried material, the values for degradability obtained from dried material may not be truly representative for its feeding value (Wight, 2006). Maize genotypes differing in starch structure (i.e. amylose or amylopectin) and composition (i.e. amylose: amylopectin) may show different responses to oven-drying, and hence the estimation of their feeding values may be influenced differently by sample processing (Haros and Suarez, 1997).

The aim of the present study was to investigate the influence of forced oven-drying on the starch degradability of maize kernels *in vitro* using rumen fluid. Although, kernels differing in maturity, obtained from different genotypes and from plants grown under different temperature regimes during grain filling were investigated, this study only reports the influence of oven-drying on the rumen fermentation of the kernels.

2. Materials and methods

2.1. Maize kernel samples

Maize kernels were collected from two greenhouse experiments conducted in 2008 and 2009 at the experimental greenhouse facilities of Wageningen University and Research Centre (UNIFARM) in Wageningen, The Netherlands. Each experiment was done with six different genotypes and three growing temperature regimes during grain filling (reproductive phase) in triplicate. The genotypes were selected on the basis of variation in their amylose contents and type of endosperm (i.e. vitreousness). Genotypes used in both experiments were: homozygous dent, homozygous flint, high amylose (50% amylose) and waxy (only amylopectin). Counter parts of high amylose and waxy were used only in Exp. 1, whereas a vitreous and a non-vitreous genotype were used only in Exp. 2. Both counter parts of high amylose and waxy had an amylose content of 20-30%, but were from different parental inbred lines. The day/night temperature treatments were 18/12, 24/18 and 30/24 °C with 12h day and 12 h night in Exp. 1, and 22/12, 27/17 and 32/22 °C with 15 h day and 9 h night in Exp. 2. Cobs were harvested and kernels were manually removed from the cobs using a sharp knife. The samples were collected when starch content in the kernels was between 399-526 g/kg (350-500 °Cd accumulated thermal time, i.e. 24-43 days after pollination) and between 566-643 g/kg organic matter (OM) (500-700 ºCd accumulated thermal time, i.e. 34-63 days after pollination) in Exp. 1. In Exp. 2, the kernels were harvested at a starch content of 409-527 g/kg (300-400 °Cd accumulated thermal time, i.e. 22-29 days after pollination) and 541-638 g/kg OM (550-750 °Cd accumulated thermal time, i.e. 37-55 days after pollination) in Exp. 2. These samples were collected from one plant per replication in Exp. 1 and from two plants per replication in Exp. 2. Samples were divided into two sub-samples with one subsample immediately stored at -20 °C after harvest, whereas the second subsample was dried at 70 °C for 48 h in a forced ventilation oven and stored at room temperature. Both the fresh and dried samples were ground over a 1 mm sieve using a centrifugal mill (Retsch ZM 100, Haan, Germany). The fresh samples were ground frozen after freezing in liquid nitrogen.

The geometric mean diameter of ground maize kernels for five dried and corresponding fresh samples from five genotypes used in Exp. 2 grown under medium temperature (27/17 °C) was analysed by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Malvern, United Kingdom). Each sample was measured 5 times to provide a geometric mean value, with the mixing speed set at 1800 rpm.

2.2. Chemical analyses

The dry matter content was determined gravimetrically by drying for 4 h at 103 °C (ISO 6496), and ash was determined by incineration for 3 h at 550 °C (ISO 5984). Starch content was determined using the amyloglucosidase method described by Keppler and Decker (1970), after unsealing the starch in dimethyl sulfoxide (DMSO).

2.3. In vitro *rumen fermentation and starch degradation*

The *in vitro* fermentation of ground, fresh and dry kernels was performed using a fully automated gas production technique as described by Cone *et al*. (1996). Rumen fluid was collected 2 h after the morning feeding from two lactating rumen-cannulated cows fed with grass (2/3) and maize silage (1/3) and concentrate according to requirements. The dry matter content of ground kernels was determined before the *in vitro* incubations. Samples of 0.5 g dry matter (DM) (dry), or the equivalent of 0.5 g DM (fresh), were incubated in duplicate in 60 ml buffered rumen fluid in 250 ml bottles in a shaking water bath at 39 °C and gas production was recorded for 72 h, as described by Cone *et al*. (1996). Results were corrected for gas production in buffered rumen fluid without sample.

Based on the volume of gas production and starch content, starch degradation was calculated for 6, 12 and 20 h of incubation in rumen fluid, using the equation of Chai *et al*. (2004):

Starch degradation at time t (g/kg OM) = -191.6(±14.6) + 0.303(±0.025) × Starch content (g/kg OM) + 1.648(±0.053) × Gas produced at t (ml/g OM)

and the equation:

Starch degradation (g/kg starch) = [Starch degradation (g/kg OM) / Starch content (g/kg OM)] × 1000

2.4. Statistical analysis

The following model was used to determine the effect of drying on starch degradation (6, 12 or 20 h incubation) of different maize genotypes grown under different temperature regimes:

$$Y_{ijk} = \mu + G_i + T_j + Oven\text{-}drying_k + R_l + G_i \times T_j + G_i \times Oven\text{-}drying_k + G_i \times R_l + T_j \times Oven\text{-}drying_k + T_j \times R_l + G_i \times T_j \times Oven\text{-}drying_k + G_i \times T_j \times R_l + T_j \times Oven\text{-}drying_k \times R_l + \varepsilon_{ijk}$$

where

Y_{ijk} = starch degradation at 6, 12 or 20 h;

μ = overall mean;

G_i = genotype effect;

T_j = temperature effect;

$Oven\text{-}drying_k$ = oven-drying effect / Fresh or dried kernels;

R_l = replication effect;

ε_{ijk} = general error term.

Statistical analysis was performed using the GLM procedure of SAS (version 9.2) and means were considered statistically significant at $P<0.05$. A paired t-test was used to determine differences in the geometric mean particle size between dried and fresh ground maize kernels.

3. Results

There was a significant ($P<0.05$) difference in mean particle size distribution between the fresh and dried, ground maize kernels. The mean particles

size for the dried kernels was 286.3 μm while a higher value of 363.9 μm was found for the maize kernels frozen during grinding.

Oven-drying significantly ($P<0.001$) reduced *in vitro* rumen starch degradation at all incubation times, at all four different maturity stages and for all temperature treatments in both experiments (Tables 1 and 2), although in some case these differences were small. In Exp. 1, the average difference in starch degradation between fresh and oven-dried samples ranged from 40 to 48 and from 48 to 72 g/kg starch for the various incubation times at lower and higher starch levels (Table 1), respectively. Similarly, in Exp. 2, these differences ranged from 37 to 69 g/kg starch at lower starch levels, and from 53 to 100 g/kg starch at higher starch levels (Table 2).

The effects of growth temperature during grain filling and of genotype on starch fermentation in rumen fluid were also significant ($P<0.001$) and consistent at all incubation times. However, there was no interaction between temperature during grain filling and genotype (Tables 1-2). Higher *in vitro* rumen starch degradation values were recorded at intermediate growth temperatures (24/18 in Exp.1 and 27/17 °C in Exp. 2, Tables 1 and 2), whereas the lowest values were observed at lower (18/12 °C, Table 1) and higher growth temperature (32/22 °C, Table 2) in Exp. 1 and 2, respectively.

Genotypes significantly affected fresh and oven-dried *in vitro* rumen starch degradation of maize kernels in the same pattern except for high amylose. The highest values for starch degradation were observed for waxy and non-vitreous, whereas the lowest values were found for high amylose in Exp. 1 (Table 1) and vitreous in Exp. 2 (Table 2). The results showed no significant difference between waxy and its counterpart, but a significant difference between high amylose and its counterpart (Table 1).

The interaction between oven-drying (dry and fresh) and genotype was significant at all incubation times and all maturity stages in both experiments (Table 1 and 2). Oven-drying showed a greater impact on high amylose maize than all other genotypes (Table 1 and 2). No significant interaction between oven-drying and genotype was found when data analysis was conducted without the high amylose

Table 1
Influence of oven drying on ruminal starch degradation at three different incubation times (6, 12 and 20 h) of kernels of different maize genotypes grown at different temperatures during grain filling and harvested at two different starch content levels (368-633 and 558-674 g/kg OM) in Exp. 1.

Temperature (T, °C) day/night	Genotype (G)	Ruminal starch degradation (g/kg starch)													
		Starch contents 368-633 g/kg							Starch contents 558-674 g/kg						
		6 h		12 h		20 h		Pooled SEM	6 h		12 h		20 h		Pooled SEM
		Dry	Fresh	Dry	Fresh	Dry	Fresh		Dry	Fresh	Dry	Fresh	Dry	Fresh	
18/12	Dent	380	398	689	720	782	818	20.5	243	317	568	613	649	679	35.3
	Flint	334	351	626	653	725	770	25.6	200	255	534	571	611	644	30.0
	High amylose	290	352	571	638	667	739	33.7	133	208	461	535	554	611	38.9
	High amylose CP	339	397	702	722	767	806	13.9	232	303	575	618	644	712	22.8
	Waxy CP	370	418	713	740	789	826	20.5	248	320	590	642	662	700	32.2
	Waxy	394	401	720	748	795	833	22.7	255	325	603	654	669	705	33.3
24/18	Dent	418	463	735	765	832	852	16.5	272	368	597	657	698	759	27.4
	Flint	371	428	685	722	790	812	17.8	248	296	572	619	672	688	22.8
	High amylose	339	401	632	690	733	788	34.8	174	265	499	596	600	678	35.5
	High amylose CP	416	465	734	753	807	848	23.0	299	354	620	672	723	756	32.0
	Waxy CP	424	468	743	784	831	891	20.5	301	382	629	690	730	781	28.7
	Waxy	441	470	748	792	827	890	15.0	305	393	619	698	734	787	27.5
30/24	Dent	385	423	706	746	804	843	18.2	264	359	577	632	669	719	38.3
	Flint	325	384	643	681	744	793	21.2	209	266	531	599	632	668	20.6
	High amylose	284	366	602	653	691	753	29.6	142	221	484	561	565	639	45.4
	High amylose CP	393	418	716	742	816	836	20.5	276	335	609	644	707	741	24.4
	Waxy CP	402	440	726	763	813	850	25.5	279	366	608	651	707	751	33.6
	Waxy	404	440	734	776	809	859	24.6	300	380	615	665	712	774	38.3
P-value															
Dried *vs.* fresh		<.0001		<.0001		<.0001			<.0001		<.0001		<.0001		
	Dried	373[b]		690[b]		779[b]			243[b]		572[b]		663[b]		
	Fresh	416[a]		727[a]		823[a]			317[a]		629[a]		711[a]		
T		<.0001		<.0001		<.0001			<.0001		<.0001		<.0001		
	18/12	369[c]		687[c]		776[c]			253[c]		580[c]		654[c]		
	24/18	425[a]		732[a]		825[a]			305[a]		622[a]		717[a]		
	30/24	389[b]		707[b]		801[b]			283[b]		598[b]		690[b]		

G		<.0001			<.0001			<.0001			<.0001			<.0001			<.0001		
	Dent	411^{bc}			727^{c}			822^{b}			304^{c}			607^{c}			696^{c}		
	Flint	365^{d}			668^{d}			772^{d}			246^{d}			571^{d}			653^{d}		
	High amylose	339^{e}			631^{e}			728^{e}			191^{e}			523^{e}			608^{e}		
	High amylose CP	405^{c}			728^{c}			813^{c}			300^{c}			623^{b}			714^{b}		
	Waxy CP	420^{ab}			745^{b}			833^{a}			316^{b}			635^{a}			722^{ab}		
	Waxy	425^{a}			753^{a}			836^{a}			326^{a}			642^{a}			730^{a}		
Drying × T		0.1256			0.5499			0.9532			0.5266			0.0726			0.5507		
Drying × G		0.0022			0.0043			0.0081			0.0134			0.0088			0.0058		
	Dent	394	428		710	744		806	838		260	348		581	634		672	719	
	Flint	343	388		651	685		753	792		219	272		545	596		638	667	
	High amylose	304	373		602	660		697	760		150	231		481	564		573	643	
	High amylose CP	383	427		717	739		796	830		269	330		601	644		691	736	
	Waxy CP	398	442		727	762		811	856		276	356		609	661		700	744	
	Waxy	413	437		734	772		810	861		287	366		612	672		705	756	
T × G		0.1942			0.0732			0.1136			0.0591			0.7464			0.0603		
Drying × T × G		0.0948			0.8441			0.1317			0.8717			0.6713			0.1153		

Means followed by different letters are significantly different (P<0.05).
OM: Organic matter; CP, Counterpart.

Table 2
Influence of oven drying on ruminal starch degradation at three different incubation times (6, 12 and 20 h) of kernels of different maize genotypes grown at different temperatures during grain filling and harvested at two different starch content levels (401-618 and 537-695 g/kg OM) in Exp. 2.

Temperature (T, °C) day/night	Genotype (G)	Ruminal starch degradation (g/kg starch)												
		Starch contents 401-618 g/kg							Starch contents 537-695 g/kg					
		6 h		12 h		20 h		Pooled SEM	6 h		12 h		20 h		Pooled SEM
		Dry	Fresh	Dry	Fresh	Dry	Fresh		Dry	Fresh	Dry	Fresh	Dry	Fresh	
18/12	Dent	375	405	682	744	741	805	24.5	258	303	597	671	663	755	36.9
	Flint	333	357	669	696	713	774	20.7	239	277	563	618	649	734	31.5
	High amylose	277	346	580	660	668	747	32.7	163	238	499	598	586	704	49.8
	Waxy	374	409	720	750	773	820	17.4	299	346	630	701	684	784	38.2
	Non-vitreous	390	416	722	751	774	818	17.2	291	347	633	701	689	788	38.5
	Vitreous	272	296	555	587	640	692	22.4	139	171	453	517	537	626	33.8
24/18	Dent	399	436	728	741	767	835	27.7	315	337	616	683	675	776	36.4
	Flint	354	387	693	721	750	783	21.2	267	318	579	656	665	762	39.3
	High amylose	302	359	619	687	680	771	38.5	195	271	513	632	605	728	54.5
	Waxy	411	453	733	773	782	857	19.9	328	377	646	718	705	810	40.0
	Non-vitreous	412	448	740	773	783	857	18.3	332	381	648	724	705	815	41.7
	Vitreous	292	315	590	615	669	728	19.7	174	204	493	557	575	642	28.9
30/24	Dent	360	384	657	712	727	785	23.6	239	272	574	635	632	714	31.9
	Flint	314	334	630	654	694	757	17.1	228	252	546	608	608	689	30.4
	High amylose	275	330	591	617	642	733	37.5	146	217	484	569	569	676	45.1
	Waxy	380	403	702	734	759	802	28.5	273	308	611	686	665	753	35.4
	Non-vitreous	389	409	707	740	760	803	26.2	279	317	616	684	671	754	33.5
	Vitreous	254	277	564	581	591	657	22.3	127	166	418	476	502	580	31.2
P-value															
Dried *vs.* fresh		<.0001		<.0001		<.0001			<.0001		<.0001		<.0001		
	Dried	342[b]		660[b]		717[b]			238[b]		562[b]		632[b]		
	Fresh	376[a]		697[a]		779[a]			283[a]		635[a]		727[a]		
T		<.0001		<.0001		<.0001			<.0001		<.0001		<.0001		
	18/12	354[b]		676[b]		747[b]			256[b]		599[b]		683[b]		
	24/18	381[a]		701[a]		772[a]			291[a]		622[a]		705[a]		
	30/24	342[c]		658[c]		726[c]			235[c]		576[c]		651[c]		

G		<.0001		<.0001		<.0001		<.0001		<.0001		<.0001
	Dent	393[b]		711[b]		777[b]		287[b]		629[b]		702[b]
	Flint	346[c]		677[c]		745[c]		263[c]		595[c]		684[c]
	High amylose	315[d]		626[d]		707[d]		205[d]		549[d]		645[d]
	Waxy	405[a]		735[a]		799[a]		322[a]		665[a]		734[a]
	Non-vitreous	410[a]		739[a]		799[a]		324[a]		667[a]		737[a]
	Vitreous	284[e]		582[e]		663[e]		163[e]		486[e]		577[e]
Drying × T		0.0853		0.1676		0.4230		0.2672		0.2424		0.0661
Drying × G		0.0002		0.0124		0.0093		0.0003		0.0052		0.0048
	Dent	378	409	689	732	745	808	271	304	596	663	657 748
	Flint	333	359	664	690	719	772	245	282	563	627	641 728
	High amylose	285	345	597	655	663	750	168	242	499	600	587 703
	Waxy	388	422	718	753	772	826	300	344	629	702	685 783
	Non-vitreous	397	424	723	755	773	826	301	348	633	703	688 786
	Vitreous	273	296	570	595	634	692	147	180	454	517	538 616
T × G		0.0759		0.0519		0.1258		0.2651		0.0623		0.3398
Drying × T × G		0.9183		0.0579		0.2271		0.6325		0.8721		0.7984

Means followed by different letters are statistically significantly different (P<0.05).
OM: Organic matter; CP, Counterpart.

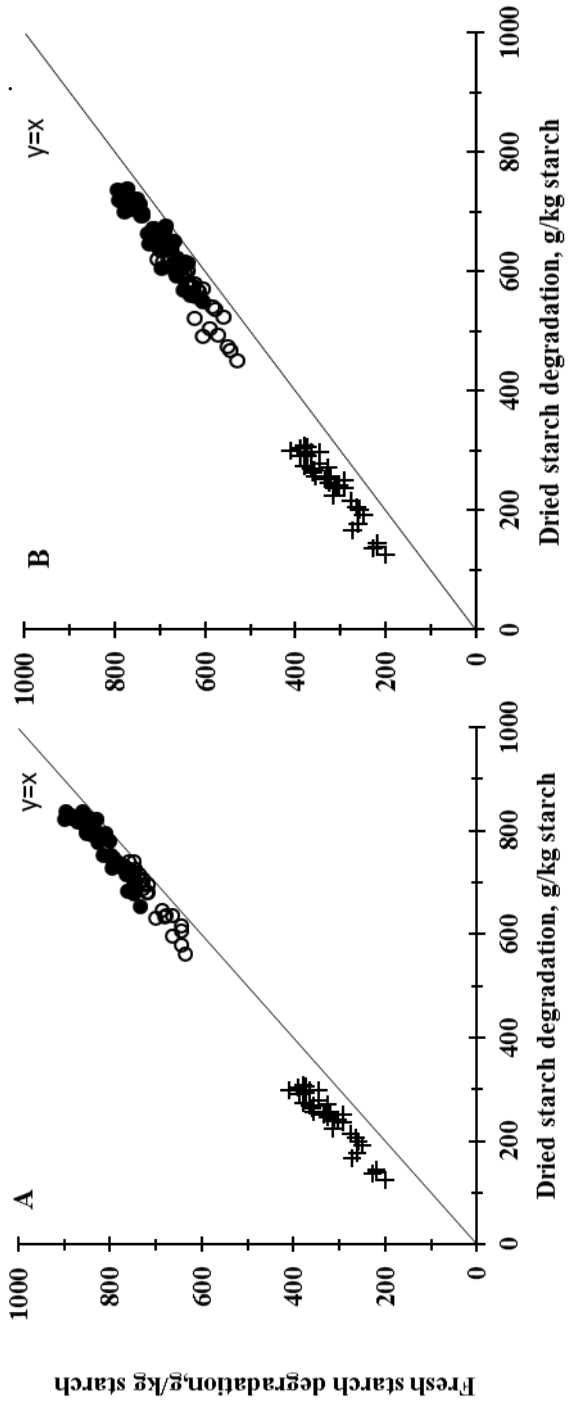

Fig. 1. Relationship between degradation of starch of fresh and dried maize kernels harvested at starch contents of 399-526 (panel A) and 566-643 (panel B) g/kg DM after 6 (+), 12 (○) and 20 (●) h of incubation in rumen fluid in Exp. 1

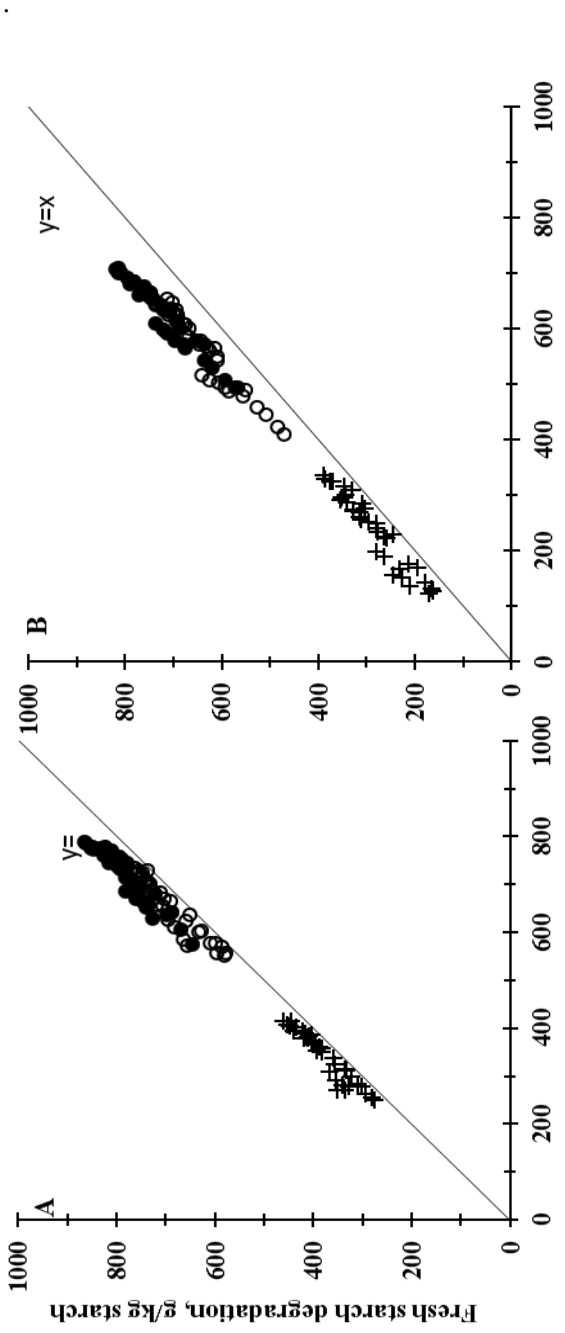

Fig. 2. Relationship between degradation of starch of fresh and dried maize kernels harvested at starch contents of 409-527 (panel A) and 541-638 (panel B) g/kg DM after 6 (+), 12 (○) and 20 (●) h of incubation in rumen fluid in Exp. 2

genotype (data not shown). The vitreous genotype showed much lower starch degradation than the non-vitreous genotype but did not show a large effect of oven-drying (Exp. 2, Table 2).

Figures 1 and 2 show the relationship between the fermentation of fresh maize starch and oven-dried maize starch. Fresh maize kernels consistently showed higher starch degradation at all incubation times, regardless of genotype, growing condition and maturity stage, although in some cases differences were small. In Exp. 1, the oven-drying of kernels seemed to have a greater impact at 6 h of incubation, especially at higher starch content levels, than at 12 and 20 h of incubation in rumen fluid (Fig. 1). However, the opposite effect appeared to be the case at both starch content levels in Exp. 2 (Fig. 2). The maize used in Exp. 2 showed a wide range of difference between oven-dried and fresh kernels at all incubation times and starch contents except for 6 h incubation at higher starch levels (Fig. 2).

4. Discussion

Oven-drying significantly influenced the *in vitro* rumen starch degradation of maize kernels independent of growing conditions and starch type. Ruminal starch degradation of fresh maize kernels was higher than that of the oven-dried samples at the various incubation times. This consistent difference (although small) is most likely caused by the impact of oven-drying on the structure of the starch molecules influencing the availability and degradability of starch in rumen fluid (Sadeghi and Shawrang, 2008). The present study shows that the effect of oven-drying applies to genotypes of maize, different in starch structure (i.e. amylose or amylopectin) and composition (i.e. amylose: amylopectin) grown at different temperatures and harvested at different stages of maturity.

Starch degradation of oven-dried kernels may also be lower than that of fresh kernels because in rumen fluid the dried kernels have to be hydrated before microbial enzymes can start to degrade the starch. Fresh kernels were not dehydrated and further hydration in rumen fluid would result in a shorter lag phase before degradation begins (Canizares *et al*., 2011).

Another potential reason for the reduced *in vitro* starch degradation in oven-dried kernel could be the occurrence of the Maillard reaction, which reduces the feeding value. The Maillard reaction largely depends on moisture content and processing (drying) temperature (Ong and Law, 2010). The reaction slows down at very high moisture contents due to a dilution effect of reactants concentration. It also slows down at very low moisture contents because of limited solute mobility. In both experiments, the effect of oven-drying was larger at the more mature stage of development of the kernels, i.e. when the moisture contents were lower and the starch contents higher when the starch molecules on average are probably longer. Oven-drying can also change the starch structure of the maize kernels, resulting in more coarse material when mechanically processed. This causes less surface area to be exposed to microbial enzymes and ultimately reduces degradability (Sadeghi and Shawrang, 2008).

Oven-drying can also influence the molecular conformation of starch and its physicochemical properties (Zhao *et al*., 2007) making it less degradable. Oven-drying can cause retro-gradation of the amylose macro-molecules. During retro-gradation the linear amylose macromolecules are rearranged parallel and form crystalline structures, which are less degradable (Rooney and Pflugfelder, 1986; Maheri-Sis *et al*., 2011). Oven-drying of the high amylose genotype maize kernels showed a much larger effect on starch degradation in rumen fluid than the other genotypes. High amylose showed the lowest ruminal starch degradation, both in the fresh and dry samples, but the effect of oven-drying compared to fresh, was larger than in the other genotypes. This supports the fact that amylose is more susceptible to retro-gradation and forms more crystalline regions than the highly branched amylopectin. The high amylose genotype has higher amylose content than the other genotypes (Sadeghi and Shawrang, 2006). The crystalline starch structure is the most important factor determining its degradability as shown by Zhang and Oates (1999) and Wolters and Cone (1992), as this makes it less accessible to enzymes (Vasanthan and Bhatty, 1996). Moreover high amylose starch has a lower water binding capacity than amylopectin. This could be due to the presence of more hydrogen bonds between and within the linear amylose molecules, which results in

reduced availability of hydroxyl groups to bind water (Wootton and Bamunuarachchi, 1978). This may reduce the rumen starch degradation. Perhaps this could be the reason of the large effect of oven-drying on starch degradation in kernels from high-amylose maize.

The poorly degradable vitreous genotype responded similarly to the oven-drying compared to the non-vitreous genotype, despite the fact that the enzymatic degradation rate and accessibility are reduced in the denser and more complex network of the vitreous types (Cui and Oates, 1999; Svihus *et al*., 2005) resulting in poor rumen starch degradation (Holm *et al*., 1983; Vasanthan and Bhatty, 1996).

The present study reports a significant effect of only oven-drying on *in vitro* ruminal starch degradation, but the impact of different storage conditions and grinding procedures of fresh and oven-dried kernels should not be ignored. The fresh kernels were stored at -20 °C and were ground frozen while the dried samples were stored and ground at room temperature. Although these differences in treatment can be expected to have little impact compared to the oven-drying of the kernels, it cannot be excluded that these differences may have had some effect on the *in vitro* rumen degradability of the starch. Differences were observed in the geometric mean particles size between fresh and dried ground kernels and this may have affected the starch degradation to some extent. Whether the difference in particle size significantly contributed to the observed differences in starch degradation remains to be determined.

Current feed evaluation systems use data, based on analysis of dried kernels to evaluate feeds and feed ingredients. The present study indicates that fresh starch shows significantly higher ruminal starch degradation than similar dried starch. In addition, genetic improvement programs for plants use NIRS data which have been calibrated using oven-dried kernels. The latter may underestimate starch availability in the rumen and incorrectly assess genetic variation in starch degradation. As such, not only feed evaluation systems but also plant breeding companies should take into account the difference between fresh and oven-dried starch.

5. Conclusion

Oven-drying of maize starch kernels significantly reduced the starch degradability in rumen fluid, compared to non-dried fresh maize kernels. This lower rumen starch degradability was observed at various incubation times in rumen fluid, and at different growing conditions and maturity stages of the maize plants. The largest effect of oven-drying was seen for the high amylose genotype. Evaluating the feeding value of starch in maize kernels after oven-drying will provide an underestimation of the true value. Oven-drying will also obscure genetic variation in starch degradation.

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Ruminal degradation of maize silage is influenced by temperature and duration of ensiling

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Chapter 5

ABSTRACT

The effects of ensiling temperature and duration of ensiling on *in vitro* fermentation of maize (*Zea mays* L.) silages were investigated. Samples of one cultivar of maize plants were collected from two plots, grown in different years on sandy soils. Samples were collected when the whole-plant dry matter content was approximately 330 g/kg. Maize plants were chopped and ensiled in mini silos at three different temperatures (5, 12 and 18 °C). Samples from the silos were taken after 0 (not ensiled, i.e. control), 4, 8 and 16 weeks of ensiling. Fresh silage samples were ground frozen under liquid nitrogen to pass a 1-mm sieve. The gas production technique was used to evaluate the influence of the ensiling temperature and duration of ensiling on the degradation of the silage samples in rumen fluid. The final pH of the silages was lowest and the gas production was highest when the maize was ensiled at 12 °C during both years ($P<0.0001$). Gas production and silage pH decreased with an increase in ensiling duration ($P<0.0001$). The decrease in pH was linearly related to the decrease in gas production in rumen fluid at the three temperatures. The ensiling temperature affected the ensiling fermentation processes, causing different rates of pH decline and different final pH values. Through the same mechanism, temperature also affected the fermentation in rumen fluid. The present study concludes that ensiling temperature, through its effect on rate of change of pH, and ensiling duration, determine the final pH, and play a significant role in the *in vitro* fermentation of maize silage.

Keywords: Maize (*Zea mays* L.) silage, Ensiling temperature, Ensiling duration, Forage quality, Gas production, Ruminants.

1. Introduction

Maize silage is used as a main component in feed rations for ruminants in many parts of the world, due to its high palatability, high energy content and relatively constant feed quality (Khan *et al.,* 2012). To produce a good silage, it is essential to have a good microbial fermentation process, which requires a rapid decline in pH to approximately 4.0 (Oude Elferink *et al.,* 2000), without significant losses of digestible dry matter. A good microbial fermentation process during ensiling results in a maximum conservation of the ensiled matter with minimum nutritional losses. The latter can occur not only during silage making, but also during storage and the feed-out period (Khan *et al.,* 2009). Some of these losses are unavoidable, like some of those through plant respiration (McGechan, 1990), while transportation losses, fermentation losses and feed-out losses can be minimized by proper management of the silages.

A good fermentation process and a rapid decline in pH not only depend on the type and quality of the forage crop, but also on the ensiling conditions (Oude Elferink *et al.,* 2000). Ensiling temperature may affect the (relative) activity of the various microorganisms involved in the fermentation process. The activity of microorganisms can be desirable (i.e., contributing to the conservation process and improving feed quality) or undesirable (i.e., increasing losses in dry matter and quality) and hence play a vital role in the ensiling fermentation and the feeding quality of the silage (Weinberg *et al.,* 2001).

Generally, active microbial processes in the silo cause a rapid decline in pH, and can take up to about 2 to 6 weeks, depending on the sugar content of the raw material and the ensiling conditions. Once the pH is around 4.0, the silage is stable for a long period, until it is opened for feeding and exposed to air (Pahlow *et al.,* 2003). Conversely, there is evidence that some microbial activity occurs during the stable phase of the ensiling process (Der Bedrosian *et al.,* 2012). Ensiling duration, therefore, may affect the final nutritional quality of the silage.

The present study was designed to elucidate how ensiling temperature and duration interact to influence the *in vitro* fermentation of maize silages.

2. Material and methods

2.1. Collecting samples and silage making

Maize with an endosperm of average vitreousness was grown on sandy soil in 2008 (Exp. 1) and 2010 (Exp. 2) on two different plots of the experimental farm of Wageningen University. Crops were tested for their dry matter content before harvesting, and were harvested when they reached a whole-plant dry matter content of approximately 330 g/kg. The crops were harvested and chopped (particle size 0.5-1.0 cm) by using a Johli Multi Hacksler (M.T. Johmann, Limbach, Germany). Control samples of chopped maize were packed in plastic bags and stored immediately at -18 °C, whereas the remaining material was ensiled in glass silos of 1-L capacity (Weck, Wehr-Oftlingen, Germany). Each silo contained 550–600 g of chopped whole-plant maize. The silage density in the silos, on a wet weight basis, was approximately 550-600 kg/m^3, i.e. slightly below standard values in practice at a dry matter content of the maize of 330 g/kg (Muck *et al.,* 2003). The silos were made air tight with rubber rings between the glass lid and bottle. Treatments (three replicate silos) comprised three ensiling temperatures (5, 12 and 18 °C), with each four ensiling durations, i.e. 0 (control), 4, 8 and 16 weeks. After the designated ensiling duration, the silos were stored at -18 °C, until analysis.

2.2. Chemical analysis

Dry matter (DM; g/kg fresh) was determined immediately after opening of the silos by drying at 70 °C in a forced ventilation oven for 48 h. These dried samples was stored and later used for further analysis. The dry matter content of the stored material was determined gravimetrically by drying for 4 h at 103 °C (ISO 6496, 1999), and ash was determined by incineration for 3 h at 550 °C (ISO 5984, 2002).

For pH measurement, 20 g of a ground maize silage sample was suspended in 100 ml distilled water and stored for 30 min at room temperature, as described in Müller and Amend (1997). Then pH was measured in duplicate for each silo and control, using a calibrated glass electrode (Mettler Toledo FE 20, Schwerzenbach, Switzerland).

2.3. Gas production technique

Fresh silage and control samples were ground over a 1-mm sieve (Retsch ZM 100, Haan, Germany) after dipping the samples in liquid nitrogen for approximately 1 min. Samples of fresh ground silage and control samples were incubated in buffered rumen fluid, using the gas production technique as described by Cone *et al.* (1996). Rumen fluid was collected 2 h after the morning feeding from two rumen cannulated lactating cows, fed to requirements. Samples were oven dried (70 °C) prior to conducting the gas production measurements to determine their dry matter contents. An amount of fresh silage sample, equivalent to 0.5 g DM, was incubated in duplicate in 60 ml buffered rumen fluid in 250 ml bottles in a shaking water bath at 39 °C. Gas production was recorded for 72 h, using a fully automated system, as described by Cone et al. (1996). Results were corrected for blank gas productions, i.e. gas production in buffered rumen fluid without a substrate. Gas production analysis was done in two replicate series for the control and the samples of 2008 and in three replicate series for the control and the samples of 2010. Each run consisted of 40 samples in duplicate. In each series a pure starch (standard) and standard maize samples (normal and gelatinized) were also incubated to allow standardisation. However, t-tests showed no significant differences between runs for these samples, making corrections not necessary.

Gas production profiles were described with a tri-phasic model (Groot *et al.*, 1996). Each sub-curve is determined by an asymptotic maximum gas production (A), the time needed to reach half of A (B) and a parameter (C) determining the shape of the curve (Cone *et al.,* 1996; Groot *et al.,* 1996). The parameter B is a measure for the rate of fermentation, a low value of B indicates a fast fermentation and a high value of B means a slow fermentation. Cone et al. (1997) showed that the gas production of the first sub-curve (A1) is caused by fermentation of the soluble fraction and that of the second sub-curve (A2) by fermentation of the insoluble fraction. The third sub-curve is caused by microbial turnover after exhaustion of the substrate and is not related to feeding value (Cone *et al.,* 1997). In the present study, values of A1 (gas produced after 3 h), A2 (the difference in cumulative gas production values between 20 and 3 h) and

B2 (half time value to reach A2), as parameter for the rate of rumen fermentation, are reported, next to the cumulative gas production after 72 h incubation.

Gas production and pH were plotted against the accumulated ensiling thermal time. The accumulated ensiling thermal time was based on temperature data obtained from the data loggers placed at the ensiling place and was calculated as:

$$Accumulated\ Thermal\ Time\ (in\ °Cd) = \Sigma\ [(Tmax + Tmin)/2]$$

Where Tmax and Tmin (in °C) are the daily maximum and minimum ensiling temperatures.

2.4. Statistical analysis

The following formula of the GLM procedure in SAS software (SAS, 2002, version 9.2) was used to calculate the effect of the ensiling temperature and duration on the pH, 72 h cumulative gas production and the gas production parameters A1, A2 and B2.

$$Y_{ijk} = \mu + T_i + D_j + R_k + T_i \times D_j + T_i \times R_k + D_j \times R_k + \varepsilon_{ijk}$$

where

Y_{ijk} = pH, 72 h gas production, A1, A2, B2;

μ = overall mean;

T_i = ensiling temperature;

D_j = ensiling duration;

R_k = replication;

ε_{ijk} = general error term.

The decline in pH and gas production was calculated for each ensiling period from initial (0 days; non-ensiled) values of the pH and gas production.

3. Results

3.1. Dry matter and ash content

After opening of the mini silos, the dry matter content was on average not significantly lower than the dry matter concentration for the control in both experiments (Tables 1 and 2). There were no relevant differences in ash content between treatments (Tables 1 and 2).

3.2. Silage pH

The pH of the maize silages declined with increasing ensiling duration and was significantly influenced by the ensiling temperature (Tables 1 and 2). The ensiling temperature also significantly influenced the time needed to reach a stable pH. The pH stayed highest when ensiling occurred at 5 °C, and was significantly lower at 12 °C than at 18 °C. The pH continued to decline with the duration of ensiling, until the end of the experimental period (16 weeks) in both experiments (2008 and 2010). The decline in pH over time (expressed in days or in thermal time) was faster for the intermediate ensiling temperature (12 °C) than for the other ensiling temperatures (5 and 18 °C) (Fig. 1).

3.2. Gas production

The ensiling temperature significantly influenced the fermentation of the maize silages in rumen fluid, as shown by the gas production, at all ensiling durations (Tables 1 and 2). Whereas the control (non-ensiled) showed the highest gas production, an ensiling temperature of 12 °C showed higher total gas productions than 18 and 5 °C, in both experiments. An increase in duration of ensiling significantly reduced the gas production of the maize silages at all ensiling temperatures (Tables 1 and 2). Ensiling temperature and duration did not show a significant interaction for total gas production (GP 72 h).

The effect of different ensiling temperatures and ensiling periods on the fermentation of the silages in rumen fluid were significant for the different gas production parameters (Tables 1 and 2). The highest A1 values were observed when

Table 1
Effects of temperature and duration of ensiling on pH, dry matter content, ash content, gas production after 72 h (GP 72 h), and the calculated gas production parameters A1 (GP 3 h), A2 (difference between GP 20-3 h) and B2 (half time value of A2) in Exp. 1 (2008).

Ensiling		DM at silo	Ash,	pH	GP 72 h,	A1,	A2,	B2,
Temperature (T), °C	Duration, wks	opening, g/kg	g/kg DM		ml/g OM	ml/g OM	ml/g OM	h
Control	0	330 ± 0.10	68 ± 8.7	6.70 ± 0.02	355 ± 2.4	77 ± 2.7	231 ± 3.0	6.6 ± 0.08
5	4	326 ± 0.45	70 ± 2.8	5.44 ± 0.07	310 ± 4.9	59 ± 5.7	192 ± 0.2	7.6 ± 0.19
	8	325 ± 1.24	61 ± 1.6	5.22 ± 0.03	300 ± 2.3	71 ± 15.5	154 ± 4.2	9.7 ± 0.07
	16	325 ± 0.59	57 ± 11.8	4.88 ± 0.07	283 ± 8.9	41 ± 1.0	191 ± 0.0	7.9 ± 0.01
12	4	328 ± 0.99	57 ± 5.5	4.42 ± 0.03	337 ± 4.0	82 ± 8.6	222 ± 4.4	6.5 ± 0.28
	8	327 ± 0.19	50 ± 0.7	4.02 ± 0.07	325 ± 3.2	67 ± 3.8	200 ± 0.0	7.3 ± 0.05
	16	325 ± 0.03	50 ± 4.6	3.65 ± 0.04	303 ± 2.8	64 ± 0.6	188 ± 14	7.5 ± 0.14
18	4	329 ± 0.63	58 ± 7.1	4.96 ± 0.04	327 ± 4.2	53 ± 1.7	234 ± 2.0	7.6 ± 0.28
	8	328 ± 1.51	50 ± 0.8	4.50 ± 0.06	313 ± 6.2	51 ± 8.4	208 ± 18	7.6 ± 0.81
	16	327 ± 1.11	50 ± 3.9	3.97 ± 0.03	298 ± 3.6	75 ± 3.2	147 ± 1.7	10.0 ± 0.24
P-value								
T		-	-	<.0001	0.0019	0.0046	0.0612	<.0001
D		-	-	<.0001	<.0001	0.0709	0.0002	<.0001
T × D		-	-	0.0002	0.5229	0.0198	0.0028	0.0002
5 °C		-	-	5.57^{a}	311^{c}	57.8^{b}	195^{b}	7.9^{a}
12 °C		-	-	4.71^{c}	332^{a}	80.1^{a}	208^{a}	6.9^{b}
18 °C		-	-	5.02^{b}	323^{b}	60.2^{b}	205^{ab}	8.0^{a}
0 weeks		-	-	6.71^{a}	356^{a}	76.5^{a}	231^{a}	6.6^{c}
4 weeks		-	-	4.94^{b}	325^{b}	64.8^{ab}	216^{b}	7.2^{b}
8 weeks		-	-	4.58^{c}	312^{c}	62.9^{b}	188^{c}	8.2^{a}
16 weeks		-	-	4.16^{d}	295^{d}	60.0^{b}	175^{c}	8.5^{a}

Means followed by different superscripts within column and the same factor are significantly (P<0.05) different.
DM: Dry matter; OM: Organic matter.
Starch degradation measurements were conducted in two runs. Gas production values of pure starch and standard samples did not differ (P>0.05) between runs.

Table 2

Effects of temperature and duration of ensiling on pH, dry matter content, ash content, gas production after 72 h (GP 72 h), and the calculated gas production parameters A1 (GP 3 h), A2 (difference between GP 20-3 h) and B2 (half time value of A2) in Exp. 2 (2010).

Ensiling		DM at silo	Ash,	pH	GP 72 h,	A1,	A2,	B2,
Temperature (T), °C	Duration, wks	opening, g/kg	g/kg DM		ml/g OM	ml/g OM	ml/g OM	h
Control	0	330 ± 0.83	54 ± 3.9	6.8 ± 0.05	350 ± 1.7	78 ± 11.3	229 ± 1.7	6.4 ± 0.08
5	4	329 ± 2.32	45 ± 3.9	5.4 ± 0.03	311 ± 1.8	61 ± 4.4	209 ± 0.8	6.1 ± 0.21
	8	327 ± 0.43	47 ± 2.5	5.3 ± 0.04	297 ± 2.9	55 ± 4.0	206 ± 1.4	6.7 ± 0.05
	16	327 ± 0.52	50 ± 0.6	4.9 ± 0.04	279 ± 2.9	55 ± 10.0	183 ± 4.9	5.2 ± 0.16
12	4	330 ± 1.70	51 ± 1.5	4.5 ± 0.02	335 ± 0.5	56 ± 6.1	227 ± 5.0	6.6 ± 0.11
	8	329 ± 0.78	45 ± 4.3	4.1 ± 0.03	317 ± 2.1	59 ± 5.3	206 ± 3.4	6.2 ± 0.20
	16	329 ± 1.10	46 ± 2.2	3.6 ± 0.04	298 ± 1.6	52 ± 3.4	207 ± 3.5	6.0 ± 0.26
18	4	331 ± 1.43	47 ± 2.5	5.0 ± 0.04	324 ± 1.9	56 ± 2.6	218 ± 3.4	6.4 ± 0.12
	8	330 ± 0.15	46 ± 1.8	4.5 ± 0.03	306 ± 2.2	55 ± 1.2	196 ± 3.9	5.7 ± 0.07
	16	329 ± 1.05	49 ± 4.9	4.1 ± 0.02	290 ± 3.0	53 ± 3.1	185 ± 11.	5.6 ± 0.16
P-value								
T		-	-	<.0001	<.0001	0.4570	0.0003	0.2087
D		-	-	<.0001	<.0001	0.0040	<.0001	0.0005
T × D		-	-	<.0001	0.0552	0.6101	0.1122	0.0291
5 °C		-	-	5.61^{a}	309^{c}	64.4^{a}	203^{b}	6.09^{a}
12 °C		-	-	4.74^{c}	325^{a}	57.8^{a}	221^{a}	6.28^{a}
18 °C		-	-	5.04^{b}	318^{b}	61.2^{a}	206^{b}	6.05^{a}
0 weeks		-	-	6.73^{a}	350^{a}	78.0^{a}	228^{a}	6.39^{a}
4 weeks		-	-	4.97^{b}	323^{b}	57.3^{b}	218^{b}	6.37^{a}
8 weeks		-	-	4.63^{c}	307^{c}	55.9^{b}	203^{c}	6.21^{a}
16 weeks		-	-	4.20^{d}	289^{d}	53.1b	191^{d}	5.58^{b}

Means followed by different superscripts within column and the same factor are significantly (P<0.05) different.

DM: Dry matter; OM: Organic matter.

Starch degradation measurements were conducted in two runs. Gas production values of pure starch and standard samples did not differ (P>0.05) between runs.

ensiled at 12 °C in Exp. 1 (Table 1), but no significant differences were observed for Exp. 2 (Table 2). Treatment 12 °C gave higher values of A2 in Exp. 2 and in Exp. 1 A2 for 12 °C was only higher than that of 5 °C (Tables 1 and 2). Control samples showed higher values for A1 than after 8-16 weeks of ensiling in Exp. 1 (Table 1) and after 4-16 weeks of ensiling in Exp. 2 (Table 2). The ensiling temperature significantly influenced B2 (half time value to reach A2) in Exp. 1 (Table 1), whereas it was insignificant in Exp. 2 (Table 2). In Exp. 1, at 12 °C ensiling temperature, it took significantly less time to reach A2 than at 5 and 18 °C (Table 1). The effect of ensiling duration was significant for B2 (Tables 1 and 2). For the control samples, it took significantly shorter (Expt. 1, Table 1) or insignificantly longer (Expt. 2, Table 2) (higher values of B2) to reach half of the maximum of A2.

Both ensiling temperature and ensiling duration did not significantly affect the curve steepness (C2) of the gas production profiles in the two experiments (data not shown). A significant interaction between ensiling temperature and duration for B2 in both experiments was observed. This interaction was also significant for A1and A2 in Exp. 1 (Table 1), but not in Exp. 2 (Table 2).

When the total gas production, after 72 h of incubation in rumen fluid, was plotted against the duration of ensiling, either expressed in weeks or in thermal time (degree-days) (Fig. 2), trends of the three ensiling temperatures differed. The lower ensiling temperature of 5 °C gave always lower values of gas production at the same ensiling duration in days and in thermal time. The gas production of the maize silages was significantly correlated with the pH of the silages. When gas production was plotted against pH over time (Fig. 3), a linear decrease were observed with the higher gas production values observed at the equivalent time points for the maize ensiled at 12 °C. The decrease in gas production (relative to the control) was linearly related with the decrease in pH for all temperatures (Fig. 4) and showed a high goodness of fit ($0.97<R^2<1.00$). At 5 °C, the highest decrease was found in gas production in both years. The rate of decrease in gas production, relative to the control, was similar between the other temperatures during 2010.

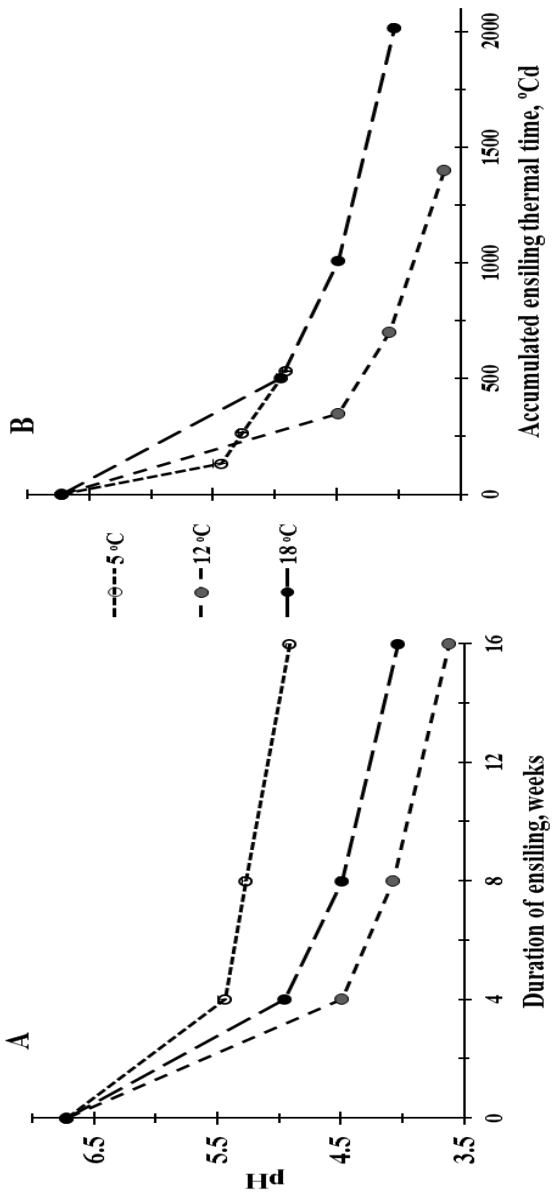

Fig. 1. pH of maize silage as influenced by ensiling duration (panel A) and accumulated ensiling thermal time (panel B), when ensiled at three different temperatures, averaged for Exp. 1 and 2.

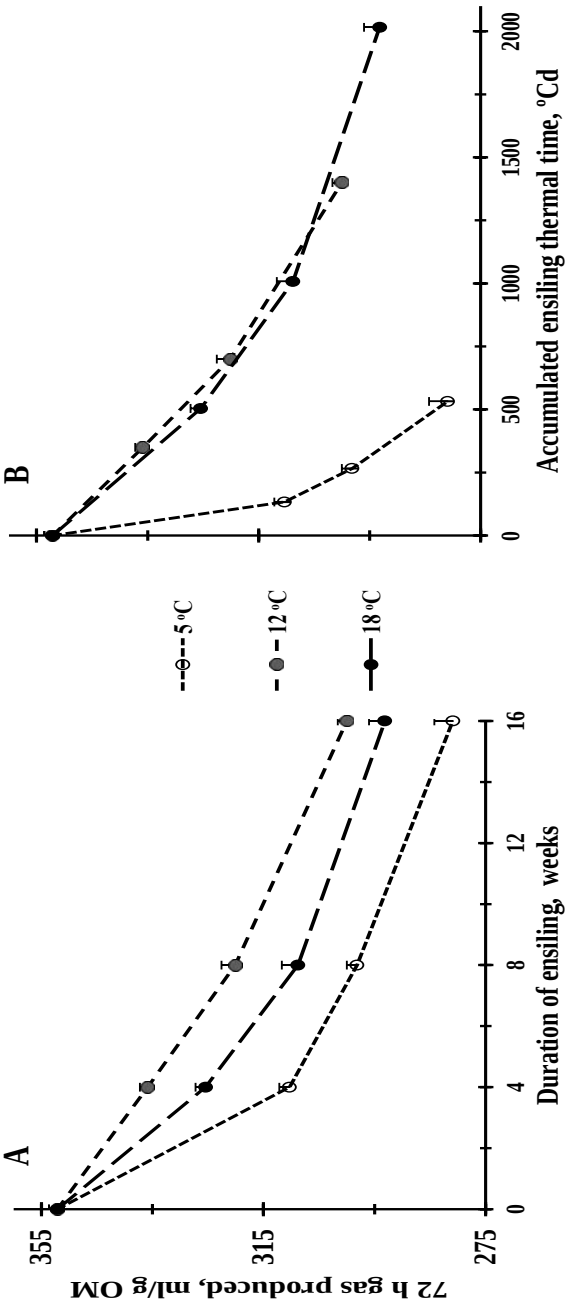

Fig. 2. Relationship between gas production (ml gas/g organic matter) caused by fermentation of the silage samples in rumen fluid and duration of ensiling (panel A), and accumulated thermal time (panel B) at three different ensiling temperatures.

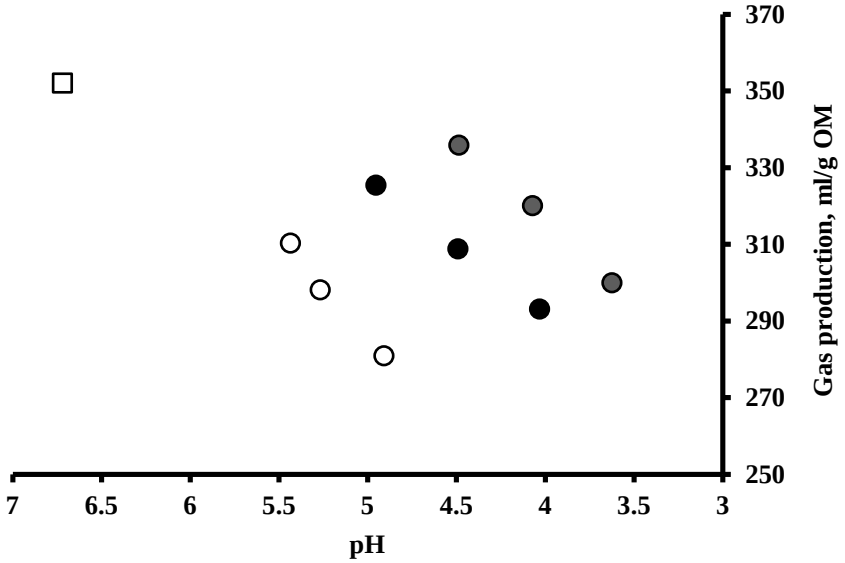

Fig. 3. Relationship between decline in pH and the decline in gas production per unit organic matter (OM) in maize ensiled for different length of time. Open, grey and solid circles are ensiled at 5, 12 and 18 °C. Square symbol represents the pH and gas production of the starting material (non-ensiled or control). Each data point is average of two experiments (2008 and 2010).

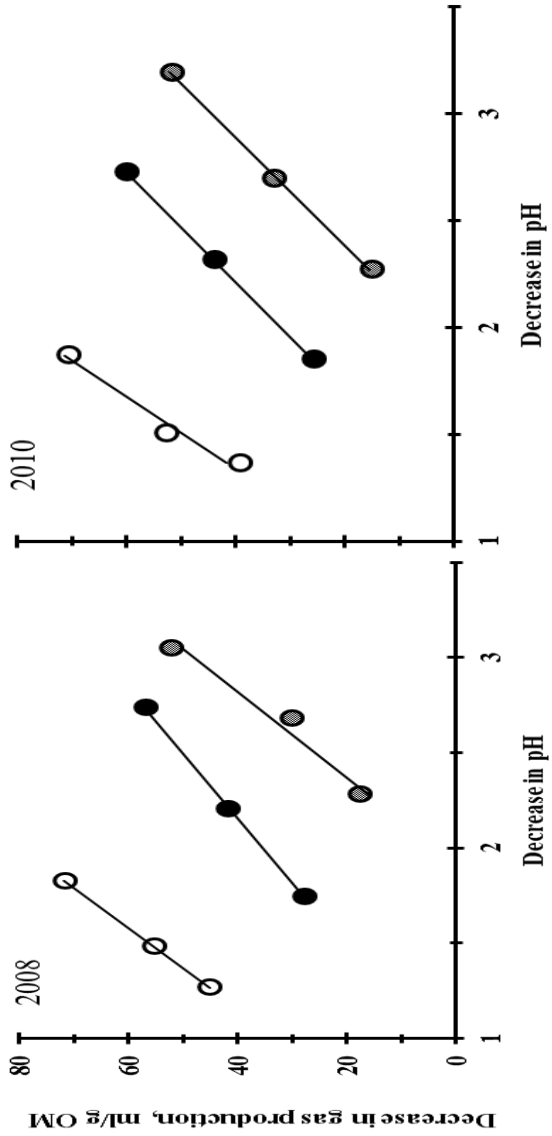

Fig. 4. Relationship between decline in pH and the decline in gas production relative to the control treatment (not ensiled) per unit organic matter (OM) in maize ensiled for different length of time in two experiments. Open, grey and solid circles are ensiled at 5, 12 and 18 °C.

4. Discussion

The absence of any significant differences in dry matter and ash content between the control and the ensiled samples illustrates the high quality of the ensiling process. However, despite the absence of differences in dry matter content, the *in vitro* rumen degradation of the maize silages was significantly influenced by the ensiling conditions, temperature and duration. Ensiling temperature and duration influenced the pH and determined the nutritional quality of the silages. Whereas control samples of maize showed higher gas production values than any of the ensiled samples, gas production was significantly influenced by the ensiling temperature and duration. The ensiling temperature of 12 °C showed the highest gas production values of A1, A2 and 72 h GP. The same ensiling temperature took less time to reach half of A2 (B2) in Exp. 1, and in Exp. 2 the ensiling temperatures were found not statistically significant for this parameter (B2). Ensiling temperatures of 5 and 18 °C showed a lower *in vitro* gas production, and hence a lower feeding value. The longer period it took to reach a low pH, the lower the gas production was. This was also associated with a comparatively slower decline in pH. The results indicate that the rate of decline in pH is also important along with the final pH of the maize silages. The higher pH (5.6) at 5 °C than normal (4.0-4.2), could be the reason of its lower degradation in our both experiments.

The final pH is a key indicator of the stability of a silage. A high silage pH indicates a poor ensiling process, resulting in a lower feed quality (Guiying *et al.*, 2000). The pH of a silage is the result of the activities of microorganisms, which can be desirable or undesirable for the ensiling process, depending strongly on the ensiling temperature (Weinberg *et al.*, 2001). To obtain a good silage, it is necessary that lactic-acid producing bacteria (LAB) predominate (Guiying *et al.*, 2000), as they are responsible for the rapid decrease in pH of the silage. A higher pH in silages is associated with low concentrations of lactic acid, because fewer LAB and more yeasts are active (Weinberg *et al.*, 2001). A high pH may also reflect the presence of other undesirable microorganisms, such as clostridia (Muck, 1988).

The lower values of gas production of the ensiled maize compared to the non-ensiled maize samples are because of fermentation losses during the ensiling

process. Ensiling is a microbial fermentation process, consuming sugars and other easily fermentable carbohydrates to produce volatile fatty acids and lactic acid. Also CO_2 is formed, which is a loss of fermentable carbohydrates. Therefore, the silage fermentation process should be as fast as possible to minimise losses. The longer the fermentation process occurs, the more undesired fermentation may take place and the less fermentable carbohydrates remain for fermentation by rumen micro-organisms (Cone *et al.,* 2008). Besides these, nutritional losses could be caused by respiration of the plants in the initial phase of ensiling, as long as oxygen is present (McGechan, 1990). Losses continue until all oxygen has been used and a low pH is reached, causing a stable phase in the ensiling process (Pahlow *et al.,* 2003).

We observed an inverse relationship between gas production and ensiling duration (up to 16 weeks) and our results are in contrast with Cone et al. (2008), who showed no influence of the ensiling period, up to 26 weeks, not on the total gas production nor on the rate of gas production. Cone et al. (2008) used oven-dried samples, whereas fresh samples were used in the present study. Another reason for this discrepancy could be the use of different laboratory silos. *Lactobacillus buchneri* can remain viable for up to 52 weeks of ensiling, even at a low pH under anaerobic conditions. Also the ammonia-N concentration can still increase after 40 weeks of ensiling (Der Bedrosian *et al.,* 2012). Moreover, sometimes prolonged ensiling results in lower concentrations of lactic acid, but higher concentrations of acetic acid, when a prolonged ensiled silage is compared with a recent silage of a few months (Kleinschmit and Kung Jr, 2006; Herrmann *et al.,* 2011). Indeed, some strains of LAB are capable of using lactic acid anaerobically when glucose is limiting (Der Bedrosian *et al.,* 2012).

5. Conclusions

Ensiling temperature and duration of ensiling influence the pH of maize silages by influencing microbial activities. The ensiling temperature significantly influences the rate of pH decline, which is vital in obtaining a stable low pH for maize silage in an appropriate time. A slower change in pH results in a reduced feeding value of the silages as measured by the *in vitro* gas production technique.

Similarly, ensiling duration along with temperature also significantly affects the pH. Prolonged periods of ensiling (up to 16 weeks) caused lower gas production values when incubated in rumen fluid.

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General discussion

Chapter 6

Background and findings

This general discussion falls into two parts according to the aims of the thesis: the first part addresses the investigation of the relationship between the rumen starch degradation of maize kernels and the broad range in structure (amylose or amylopectin), chemical composition and physical properties of starch. The interaction of these factors influencing starch degradation with growing conditions, especially temperature during grain filling, and maturity is also described. The second part of this general discussion encompasses the relationship between feeding value of maize silage and its ensiling temperature and the duration of ensiling. This part discusses how the ensiling temperature and duration influence the pH of the maize silage by influencing microbial activities which ultimately affect its feeding value.

As the maize crop is diverse in starch structure and the chemical composition of its endosperm, it is a diverse starch feed source. To address this, maize genotypes selected in this research not only reflected a wide range of structure and composition of starch but were also diverse in endosperm types. Therefore, there is a need to evaluate the feeding value of different maize genotypes and to understand factors like growing conditions, maturity stage, and post-harvest handling processes. There is also a need to understand whether there is any interaction between all these factors or not. There is a lack of scientific literature about how the interactions affect the rumen starch degradation.

A maize kernel is one of the most remarkable platforms on which genetic expression can be studied. As maize kernels contain ~61 % of starch, they play an important role in the quality of maize as an animal feed. So, there is a need to understand rumen starch degradation of maize and how it is influenced by different factors, including genotypic, environmental, and managerial factors or factors associated with sample processing. So, a database on *in vitro* rumen starch degradation characteristics of maize kernels of eight different maize genotypes contrasting in their chemical composition was developed. The *in vitro* gas production technique was used to measure the feeding quality of maize kernels and silages. Gas production data were converted into starch degradation. Chapter 3

showed the relationship between *in vitro* ruminal starch degradation of maize kernels and diversity in starch structure and composition. The same chapter also discussed the influence of environment, and genotype × environment interaction on ruminal *in vitro* starch degradation. Significant differences were found among genotypes, growth temperatures during grain filling and maturity stages in relation to *in vitro* ruminal starch degradation. The results showed that the waxy (high amylopectin) and dent genotypes as well as those low in vitreousness (floury endosperm) had higher *in vitro* ruminal starch degradation values than the genotypes which were high in amylose and vitreousness. Farmers are really concerned about the optimum maturity stage of their maize to get a better feeding value. This shows the importance of maturity stage in evaluating feeding value. Therefore rumen starch degradation at different maturity stages was also analysed and it was found that maturity significantly influenced the ruminal starch degradation. Harvesting at an earlier maturity stage showed higher ruminal starch degradation than harvesting at a later maturity stage. Growth temperature during grain filling significantly influenced the ruminal starch degradation at the same stage of maturity. The phenomena behind these observed results are explained in Chapters 2 and 3. The effects of different temperatures during grain filling on dry matter accumulation and its rate are explained in Chapter 2. Chapter 3 is about growth temperature effects on starch accumulation in a diverse set of maize genotypes based on starch structure; (i.e. amylose or amylopectin), and composition (i.e. amylose: amylopectin), and type of endosperm (i.e. vitreous and non-vitreous). It was found that a sub- or supra-optimal growth temperature during grain filling negatively influences the dry matter and starch accumulation (Chapter 2) and rumen starch degradation (Chapter 3). We did not find any kind of interaction among genotypes, growing conditions and maturity stage in our experiments. Chapter 4 further investigates the relevant methodological issue, oven-drying, affecting *in vitro* starch degradation of maize kernels using rumen fluid of lactating cows thus addressing a potential problem in quality evaluation systems. It was tested whether fresh or oven-dried maize kernels give different values for starch degradation of maize kernels when incubated in rumen fluid of lactating cows. The results showed that oven-drying, provided all other

sources of variations are the same, reduced the ruminal starch degradation in maize kernel at all incubation times and all maturity stages. Although the differences observed were small but significant, this shows the importance to use fresh kernel degradation values in feed evaluation systems in order to measure the exact feeding values of maize kernels.

Starch is a major component of maize silage providing a tremendous energy source for animal growth. It is one of the main sources of rumen fermentable energy in maize silages, which fuels the microbial activities in the rumen. But maize silage feeding value is also affected by ensiling conditions and duration, which is covered in Chapter 5 (second part of this general discussion). It is important to understand how ensiling conditions especially temperature and duration influence the maize silage feeding value. We evaluated maize silage feeding value against ensiling condition, i.e. temperature and duration; it was found that maize silage feeding value was significantly influenced by ensiling temperature and duration.

Maize: a versatile starch feed source

Maize has been an essential component in rations of lactating dairy cows throughout the world; there is a surprisingly great genetic variability within and among the diverse lines and varieties of maize in the world (Coe *et al*., 1988). Maize genotypes differ in starch structure and composition. Starch is composed of two distinct polymers, amylose (usually 20-30%) and amylopectin (usually 70-80%) (Jackson, 2003). Their relative contribution can vary among cultivars and genotypes (Fankhauser *et al*., 1989) with 100% amylopectin in waxy types and more than 50% amylose in high amylose types. Maize genotypes can also differ in endosperm type, i.e. floury (dent) vs. horny (flint) (Kotarski *et al*., 1992; Michalet-Doreau and Champion, 1995). Dent maize starch is more loosely bound in a starch:zein protein matrix and the kernel becomes indented on maturity (Fox and Manley, 2009). The flint mostly has a thick, hard, vitreous endosperm layer surrounding a small, soft granular centre (Ettle *et al*., 2001). The relative amounts of soft and corneous starch, however, vary among varieties. The vitreousness is the ratio of vitreous (hard) to floury (soft) endosperm (Fox and Manley, 2009), and is also used to assess the type

of maize endosperm. These diverse genetic variations contribute to the diversity in starch of different maize genotypes and its feeding value.

Maize silage is major feed source due to the high nutritional value of its starch, which is a fundamental component of maize silage. Starch is the most important silage feeding component because about half of the dry matter comes from the kernels and this dry matter is predominantly starch. However, huge differences in starch concentrations and its structure and composition, degradability and energy availability do exist among different maize genotypes. Those differences are based on variation in starch structure and composition along with variation in endosperm; vitreous and non-vitreous. Vitreousness is a term describing the type of endosperm in the maize kernel. Vitreousness is associated with decreased susceptibility to amylase due to increasing interaction with maize protein (Firkins, 2006). These differences influence animal performance by influencing rumen starch degradation (Philippeau and Michalet, 1997).

Ruminal starch degradation characteristics in different maize genotypes

Rumen *in vitro* starch degradation not only depends on starch degradation but also on its passage rate through rumen. Therefore, it can be defined as result of the balance between starch degradation and its passage. This means not only the rate of starch degradation but also the rates of particle and fluid passage from the rumen, influence the extent to which starch will be degraded or escapes to the lower gut (Nocek and Tamminga, 1991).

The variation in the starch structure and chemical composition of different maize genotypes results in variation in rate and extent of starch degradation in the rumen. Information on the relationship between the chemical composition and rumen starch degradation characteristics is important to estimate the feeding value of maize. Unfortunately, there is little information available in the scientific literature on the effect of variation on these relationships. We found (Chapter 3) a large variation in the *in vitro* rumen starch degradation of different maize genotypes at all incubation times. The observed variation in rumen starch degradation was due to a wide diversity in starch structure and composition among genotypes. The type

of endosperm (vitreous versus non-vitreous) also played a significant role in ruminal starch degradation. So, there was a need to understand how different contents or concentrations of amylopectin and amylose affect rumen starch degradation. To evaluate this difference, rumen starch degradation was plotted against their relative amylopectin or amylose concentrations (Figs. 1 and 2). Amylopectin and amylose contents used in figures 1 and 2 were calculated, by assuming their proportion did not change through plant development, as follows:

Amylopectin / amylose contents

= amylopectin/amylose % × starch contents at specific maturity stage

Rumen starch degradation was found positively correlated with amylopectin (Fig. 1) and negatively correlated with amylose contents (Fig. 2) for different genotypes. These relations were observed at all incubation times, growing conditions and maturity stages in both experiments. It is obvious that waxy gave higher starch degradation values than high amylose due to 100% amylopectin (Fig. 1). Moreover, as amylose contents increased from zero to 50%, the rumen starch degradation decreased (Fig. 2). This could be due to the fact that increased amylose:amylopectin ratios resulted in increased chemical interactions of amylose with lipids, resulting in reduced *in vitro* rumen starch degradation of maize (Svihus *et al.*, 2005). The effect could also be due to the crystalline structure of amylose (Sadeghi and Shawrang, 2006). This crystalline structure of amylose reduces rumen starch degradation and is possibly the most important factor in determining rumen degradability of starch (Wolters and Cone, 1992; Zhang and Oates, 1999). As waxy had only amylopectin and no amylose, this explains why waxy showed higher *in vitro* rumen starch degradation values as the high amylose genotype (Lichtenwalner *et al.*, 1978; Hibberd *et al.*, 1982). This is not only an established fact for *in vitro* (Kotarski *et al*., 1992; Opatpatanakit *et al*., 1994; Philippeau *et al.*, 1998) but also for *in vivo* (Philippeau *et al.*, 1998) degradation.

Similarly, rumen starch degradation was found inversely related to the vitreousness of the endosperm (Lichtenwalner *et al.*, 1978; Hibberd *et al.*, 1982) of

maize kernels (Chapter 3). We found higher starch degradation values for genotypes with low vitreousness (non-vitreous, dent) than for those with high vitreousness (vitreous, flint). Starch granules of vitreous starch are dense and more tightly bound, which makes them less degradable than those of non-vitreous starch. This results in reduced accessibility of starch to enzymatic degradation in vitreous genotypes (Cui and Oates, 1999; Vesterinen *et al.*, 2002; Svihus *et al.*, 2005). One can therefore conclude that the higher the vitreousness, the lower the enzymatic accessibility (Cui and Oates, 1999; Vesterinen *et al.*, 2002; Svihus *et al.*, 2005) and the lower rumen starch degradation (Holm *et al.*, 1983; Vasanthan and Bhatty, 1996) of maize kernels.

Ruminal starch degradation characteristics and different growing conditions

The results of Chapter 3 showed that not only starch content, but also structure (i.e. amylose or amylopectin) and composition of starch (i.e. amylose : amylopectin) along with vitreousness of the endosperm play an important role in ruminal starch degradation. The latter perhaps plays a more important role as shown by ruminal starch degradation values of vitreous genotypes. The results (Chapter 3) also showed that rumen starch degradation is also influenced by growing conditions (temperature) and maturity stage.

Ruminal starch degradation was significantly influenced by growing temperature, especially during the grain filling period in both experiments (Chapter 3). Rumen starch degradation could be affected by the effects of the different temperatures during grain filling on starch biosynthesis, accumulation rate and duration. The results showed that when the mean daily temperature was below 16 ºC (cooler) or above 27 ºC (warmer) during grain filling, it negatively influenced the rumen *in vitro* starch degradation. The higher starch degradation values were found when the mean daily temperature was between 20 and 27 ºC during the whole grain filling period. This difference of rumen starch degradation values at different growth temperatures could be related to the effect of temperature on dry matter (starch)

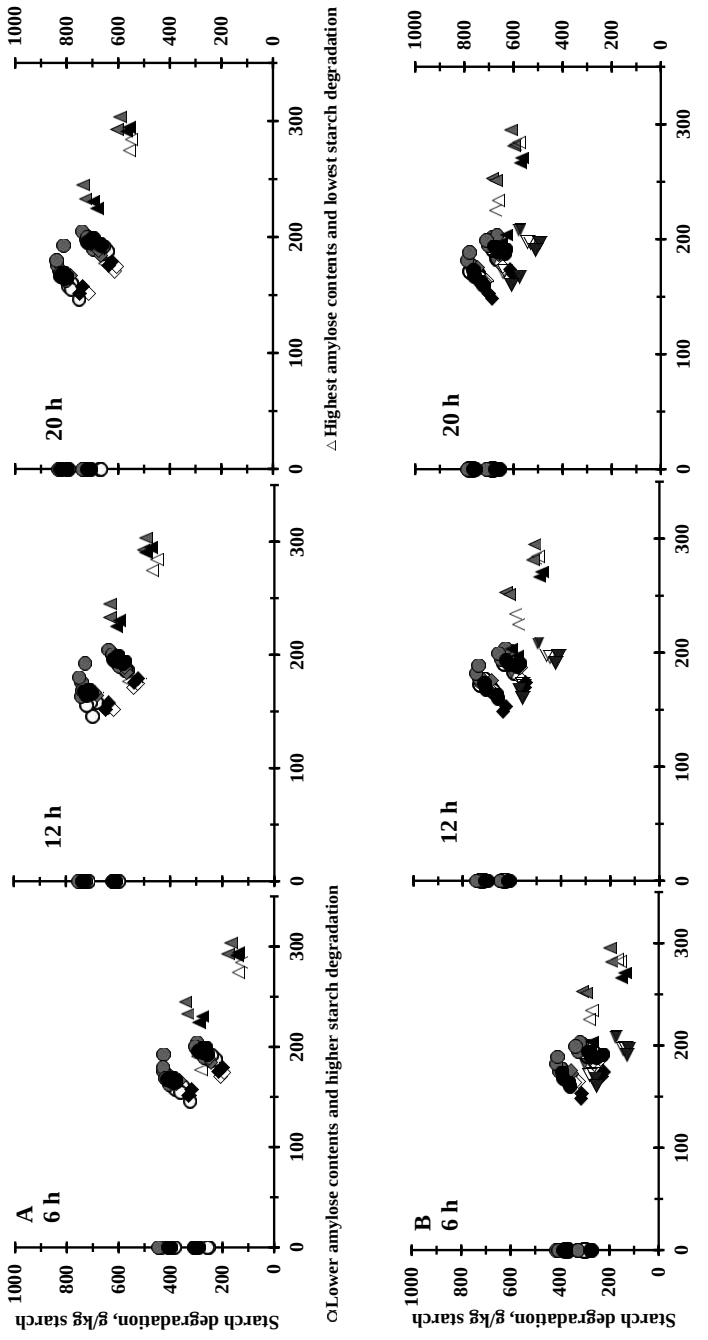

Fig. 1: Relationship between rumen starch degradation (mg/g starch) of 6, 12 and 20 h incubated maize kernels against their amylopectin contents (g/kg) at three different growth temperature treatments during grain filling in Exp. 1 (panel A) and Exp. 2 (panel B) as mentioned in Chapter 3. Lower and upper data clusters show ruminal starch degradation at higher starch contents (latest maturity stage) and lower starch contents (earlier maturity stage), respectively. Open, grey and black markers are for low (18/12, 22/12 °C), intermediate (24/18, and 27/17 °C) and high temperature (30/24 and 32/22 °C) treatments in Exp. 1 and 2, during grain filling, respectively.

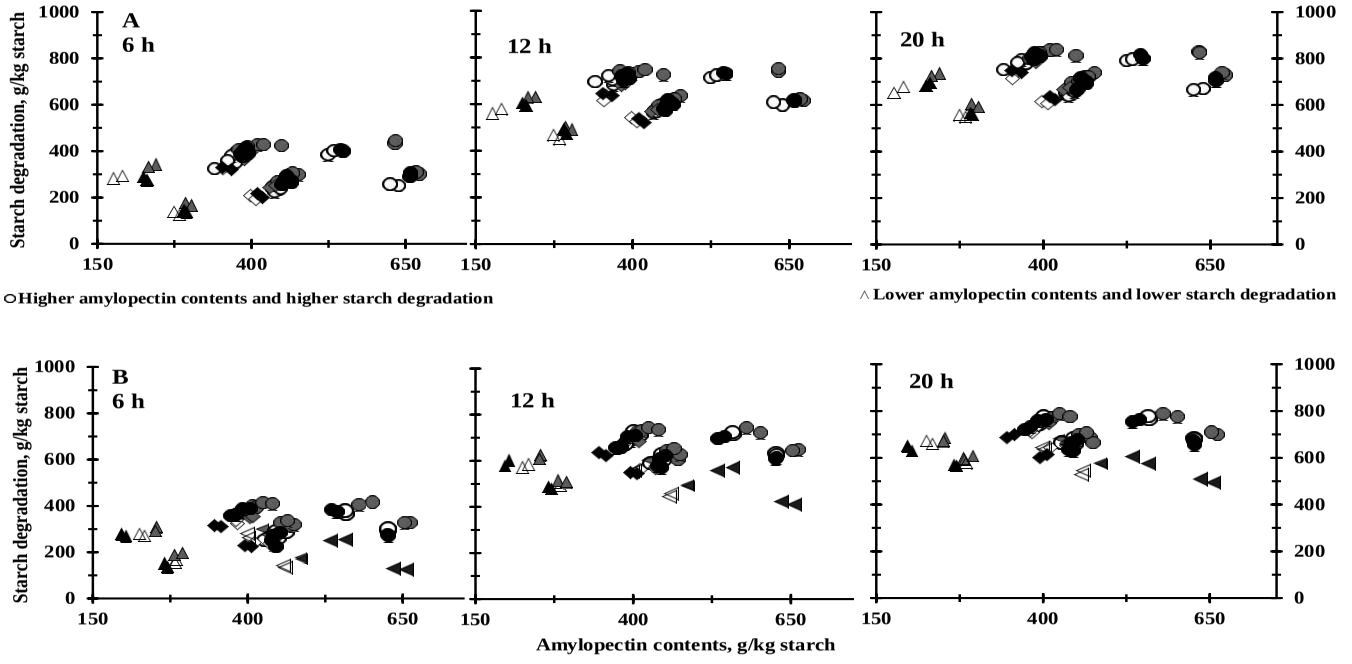

Fig. 2: Relationship between rumen starch degradation (mg/g starch) of 6, 12 and 20 h incubated maize kernels against their amylose contents (g/kg) at three different growth temperature treatments during grain filling in Exp. 1 (panel A) and Exp. 2 (panel B) as mentioned in Chapter 3 Lower and upper data clusters show ruminal starch degradation at higher starch contents (latest maturity stage) and lower starch contents (earlier maturity stage), respectively. Open, grey and black markers are for low (18/12, 22/12 ºC), intermediate (24/18, and 27/17 ºC) and high temperature (30/24 and 32/22 ºC) treatments in Exp. 1 and 2, during grain filling, respectively

accumulation, its rate and duration (Chapter 2). This indirectly influences starch biosynthesis, its accumulation rate and duration. A detailed discussion and explanation of this phenomenon can be found in Chapters 2 and 3. To fully understand how rumen starch degradation is influenced by growth temperature, we need to understand how different growth temperatures affect starch biosynthesis, accumulation rate and duration

Growth temperature effect on starch biosynthesis

As starch biosynthesis depends on enzyme activity, it is directly influenced by growth temperature. It is well known that not all enzymes involved in starch biosynthesis are equally sensitive to growth temperature. Soluble starch synthase (SSS) is probably the most temperature sensitive enzyme (Keeling *et al.*, 1993). This enzyme, along with starch branching enzymes, starch synthase 1, starch synthase 2 and starch synthase 3, is responsible for amylopectin synthesis (Tomlinson and Denyer, 2003). The activity of soluble starch synthase depends on the temperature to which kernels are exposed. It has a low temperature optimum for its activity and is also subject to heat inactivation, hence limiting starch synthesis under high temperatures, depending on the duration of exposure to heat (Rijven, 1986; Hawker and Jenner, 1993; Keeling *et al.*, 1993). Its activity ceases at 35 °C (Jenner, 1994), even then producing a high kernel weight at high temperatures, as observed in our experiments. High temperature treatments used in our two experiments were within the optimum range of soluble starch synthase (25 °C) and starch branching enzyme (27.5 °C) and did not exceed 35 °C to limit the yield.

Differences between genotypes in their response to temperature can partly be due to genetic diversification in waxy genes or differences in expression of various granule-bound starch synthase (GBSS) isoforms (Cheng *et al.*, 2005). This enzyme is responsible for amylose synthesis. Its activities are higher at high temperatures, even at temperatures up to 45 °C (Keeling *et al.*, 1993).

Growth temperature effect on dry matter and starch accumulation

As starch accounts for most of the dry matter in maize kernels, any reduction in final kernel weight and yield associated with unfavourable temperatures during grain filing is largely due to a decrease in starch content (Bhullar and Jenner, 1986; MacLeod and Duffus, 1988).

Lower dry matter yields of maize in countries with higher temperatures (e.g. 28-33 °C in Italy) can be due to higher growth temperatures (Fig. 3) than the optimum (18-19 ºC) (Wilson *et al.*, 1995). Dry matter (starch) accumulation at high temperatures is reduced. This is due to a reduced number of actively growing kernels and individual kernel weight (Fischer and Palmer, 1984; Cheikh and Jones, 1995). The number of kernels is determined between 2 weeks before and three weeks after silking (Tollenaar and Daynard, 1978; Fischer and Palmer, 1984; Andrade *et al.*, 1996). Fig. 3 shows higher maize grain yields (tonnes/hectare) in The Netherlands than in Italy, because the mean temperature in Italy is above the optimum. The mean temperature in The Netherlands during the grain filling period (July to September) is close to the optimum temperature for maize grain yield, provided the growing season is long enough. This explains why yield/ha is higher in The Netherlands than in Italy. An increase in yield can be observed when the growth temperature increases up to 23 ºC, but a further increase in growth temperature results in a reduced yield. This is because a higher growth temperature accelerates the grain filling but shortens the grain filling period (Shaw *et al.*, 1988). Moreover, a higher growth temperature shortens the other phenological phases and results in suboptimal development of the crop (Dubrovsky *et al.*, 2000). A higher growth temperature can also result in fewer kernels (Cheikh and Jones, 1994, 1995; Struik, 1983) and a shorter duration of the grain filling period (Bhullar and Jenner, 1986). Since temperature effects on rate and duration are not balanced, a higher temperature usually results in a faster maturity, but a smaller final grain size. These effects are mainly associated with changes in starch biosynthesis (Monjardino *et al.*, 2005).

Similarly cooler temperatures result in a slower accumulation of starch but an increase in duration of the grain filling period in terms of days, but not necessarily in terms of thermal time. Cultivar maturity is the best predictor of the

length of grain filling period as it defines how much thermal time is required to complete its crop cycle (Shaw *et al*., 1988). So cooler temperatures can prolong grain filling but with slower grain filling rate. When the increased grain filling duration cannot compensate for the slower grain filling rate, for example because the conditions do not allow completion of the cycle, grain dry matter accumulation remains lower. Farmers may therefore opt for earlier-maturing cultivars. This could be the reason for a lower yield of maize in Germany than in The Netherlands, (Fig. 3).The lower yields in Germany could also be, perhaps, due to the use of earlier cultivars.

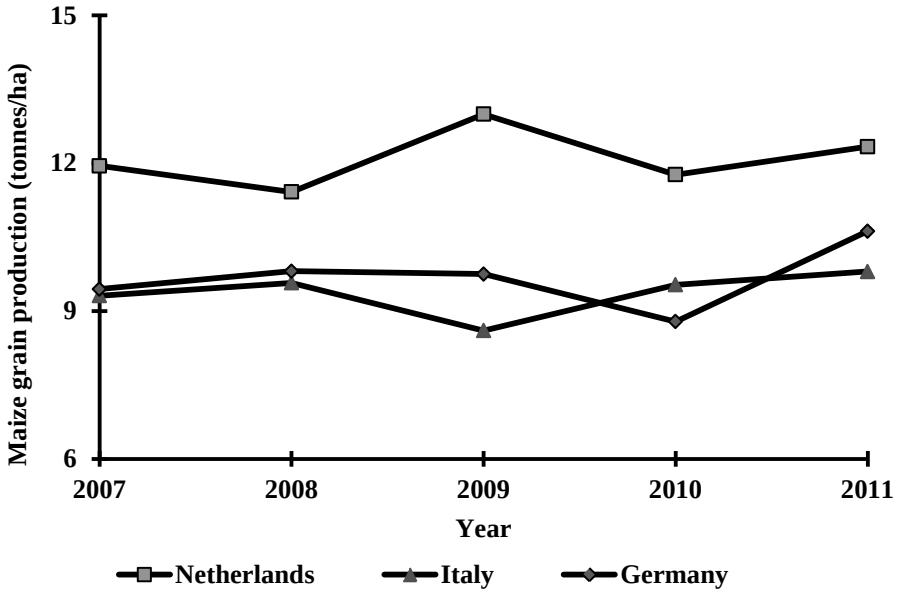

Fig. 3. FAO data of maize grain production (tonnes/hectare) during 2007-2011 in three countries differing in climate. Mean temperature for Germany, The Netherlands and Italy 16-18 ºC, 17-20 ºC and 28-33 ºC, respectively.

Rumen starch degradation and growth temperature during the grain filling period

The lower rumen starch degradation of maize, grown at the higher temperatures (Expt. 2), was caused by an impaired starch synthesis (Jenner, 1994), resulting in less starch per kernel (Tester *et al.*, 1995). Elevated temperatures also block the kernel development (Miller and Chourey, 1992; Cheng *et al.*, 1996) due to a reduced grain filling duration (Ou-Lee *et al.*, 1985; Bhullar and Jenner, 1986; Hanft and Jones, 1986). This results in less starch accumulation and lower starch contents at grain maturity (Anker-Nilssen *et al.*, 2006). Moreover supra-optimal temperatures may result in a small increase in amylose content, i.e. slower degradable starch (Anker-Nilssen *et al.*, 2006) or smaller grains with a reduced surface area for action of amyloglucosidase (Morrison *et al.*, 1986). It also results in complexes like starch–protein matrixes of the endosperm (Anker-Nilssen *et al.*, 2006) and amylose-fatty acid complexes (Tester *et al.*, 1991; Tester, 1997). These complexes reduce the rate of enzymatic degradation of starch (Cui and Oates, 1999; Crowe *et al.*, 2000) and limit ruminal starch degradation (McAllister *et al.*, 1993).

In contrast, lower rumen starch degradation values at a lower than optimum (18-19 ºC) growth temperature could be due to less starch accumulation. Wilson *et al.* (1995) found that temperatures lower than 16 ºC result in reduced crop growth rate due to inefficient use of radiation. Although it prolongs the grain filling period, but it results in less accumulation of starch and less final starch contents (Muchow, 1990; Wilson *et al.*, 1995). There is a close correlation between growing temperature and maturity of the crop (Shaw *et al*., 1988).

Rumen starch degradation and maturity

Rumen starch degradation was strongly influenced by maturity stage at which maize kernels were harvested (Chapters 2 and 3). Moisture content is very useful to predict maturity (Jensen *et al.*, 2005) and is an important tool to rank maize genotypes based on their maturity i.e. dry down and stay green (Schwab *et al.*, 2003; Marton *et al.*, 2007). However, maize genotypes can differ in their respective feeding values even at the same moisture concentration (Jensen *et al.*, 2005; Hetta *et*

al., 2012) perhaps due to differences in vitreousness, starch contents, structure and composition. The latest maturity stage showed the highest starch contents (Phipps and Weller, 1979; Argillier *et al.*, 2000; Hetta *et al.*, 2012), but ruminal starch degradation was lower as compared with earlier maturity stages (lower starch contents) in our experiments.

The lower starch degradation at later maturity stages (Cone *et al*., 2008a) could be because of an increased virtuousness of the maize kernels (Tolera *et al*., 1998; Tolera and Sundstøl, 1998; Ettle *et al.*, 2001). Increasing dry matter concentration was highly correlated with the vitreousness of the maize kernels. Advancing maturity at harvest resulted in increased vitreousness and decreased ruminal starch degradation for both flint and dent hybrids. Chapter 3 also discussed the impact of vitreousness on rumen starch degradation and why they are negatively related. Rumen degradation of starch from maize kernels by ruminal microbes is related negatively to the vitreous: floury endosperm (Philippeau *et al.*, 1999). This resistance to degradation is mainly because of the distribution of proteins in the endosperm, the concentration of zein proteins increases and of glutelin proteins decreases with increasing vitreousness. The insoluble zein proteins limit the accessibility to the starch granules by ruminal microbes compared with the soluble glutelin proteins (Philippeau *et al.*, 2000).

Moreover, with advancing maturity, sugar in the kernels is converted into starch and the moisture concentration decreases, allowing the starch granules to become more tightly packed (Allen, 2009). As is shown in Fig. 4, rumen starch degradation is a function of water content per kernel. As water content per kernel decreases, after a particular level, rumen starch degradation decreases. This prominent effect of maturity on rumen starch degradation could be due to the fact that the changes in the chemical composition of maize kernels occur up to a specific maturity stage (Struik, 1983).

Rumen starch degradation is not only influenced by genetic make-up, growing temperature, and stage of maturity at harvest, but can also be influenced by post-harvest handling of the samples during different processes. One of those

processes, that can affect starch physical properties and its conformation, can be oven-drying.

Rumen starch degradation and oven-drying

Oven-drying significantly influences the rumen *in vitro* starch degradation of maize kernels (Chapter 4). The results showed that fresh maize kernels gave a higher gas production upon fermentation in rumen fluid and so higher feed values at all incubation times, irrespective of growing conditions and maturity stages (Chapter 4). However, a significant interaction was found between genotype and the oven-drying process. High amylose showed a higher difference between starch degradation in fresh and in oven-dried samples than the other genotypes. Chapter 4 explained how the oven-drying process affected and reduced the rumen starch degradation.

Oven-drying can influence rumen starch degradation by retro-gradation of amylose macromolecules. This results in crystallisation of linear amylose molecules and crystalised molecules are less degradable (Rooney and Pflugfelder, 1986; Maheri-Sis *et al.*, 2011). This could be the reason for the significant interaction between the amylose content and oven-drying, as amylose is susceptible to retrogradation and forms crystalline regions, which amylopectin does not. The crystalline starch structure is probably the most important factor determining its degradability (Wolters and Cone, 1992; Zhang and Oates, 1999).

Oven-drying also can influence the molecular conformation of starch and its physiochemical properties (Zhao *et al.*, 2007). It can also induce the physical changes such as shrinking, crystallisation, etc. (Sagar and Kumar, 2010), making it less degradable. It can also change the starch structure of the maize kernels, resulting in more coarse material when mechanically processed. This causes less surface area to be exposed to microbial enzymes and ultimately reduces degradability (Sadeghi and Shawrang, 2008).

Rumen degradation of maize silage as influenced by its ensiling conditions and duration

Rumen degradation was significantly influenced by ensiling temperature and duration through their influence on the pH of maize silage. Both ensiling temperature and duration determined the nutritional quality of the silages (Chapter 5). We found that ensiling temperature had a significant influence, not only on the final pH, but also on the rate of decline in pH; the slower the decline in pH, the lower the feeding value. This showed the importance of an optimum ensiling temperature to maintain a proper pH decline rate for a better feeding values of maize silages. Ensiling duration influenced the rumen degradation of maize silages by affecting the final pH.

The pH plays an important role in determining the feeding quality of maize silages. The higher pH (>4.2) indicates a poor ensiling process, resulting in a low feed quality (Guiying *et al.*, 2000). The ensiling temperature significantly influences the pH of the silage through its effect on microbial activites. The microbial activites can be favourable or unfavourable for the ensiling process, depending strongly on the ensiling temperature (Weinberg *et al.*, 2001). So, any ensiling temperature would be ideal that results in a pH of 4.0 and an optimum decline rate of the pH. This is achieved when lactic-acid producing bacteria (LABs) dominate (Guiying *et al.*, 2000). LABs are responsible for the rapid decrease in pH of a silage and they have a very specific range in growth temperature (Weinberg *et al.*, 2001). A higher pH in silages is related with low concentrations of lactic acid, because less LABs means more active yeasts (Weinberg *et al.*, 2001). A high pH may also reflect the presence of other undesirable microorganisms, such as Clostridia (Muck, 1988). The results of Chapter 5 showed the importance of an optimum ensiling temperature for a better feeding value of maize silages.

Non-ensiled samples showed a higher gas production than ensiled maize samples. But the problem with non-ensiled maize is that it cannot be available throughout the year. To cope with this, ensiling is the best way to provide a valuable supplement when feed supply is not adequate. Moreover, it is suitable for a long-term storage of high quality feed. The lower gas production values of ensiled maize

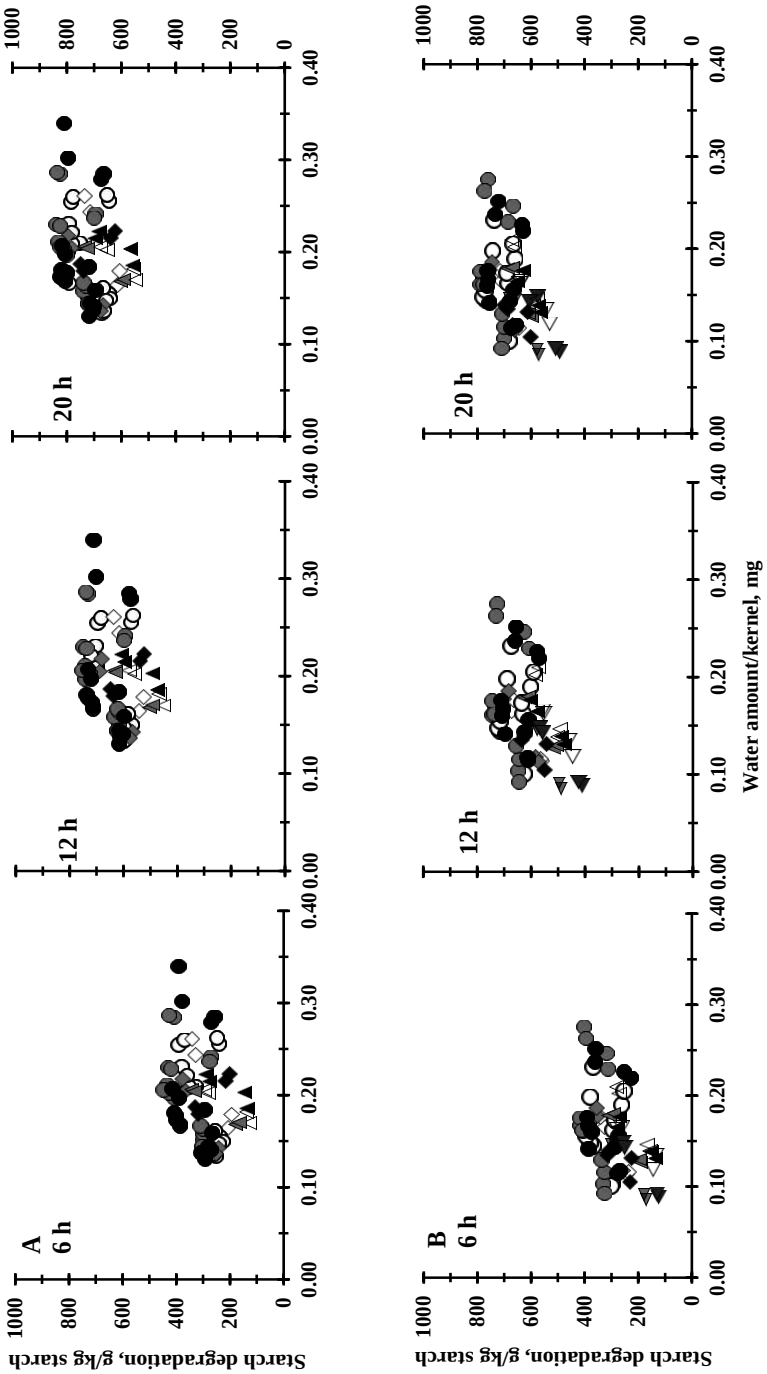

Fig. 4. Relationship between rumen starch degradation (mg/g starch) of 6, 12 and 20 h incubated maize kernels against their water contents (mg) at three different growth temperatures during grain filling in Exp. 1 (panel A) and Exp. 2 (panel B) as mentioned in Chapter 3. Open, grey and black markers are for low (18/12, 22/12 ºC), intermediate (24/18, and 27/17 ºC) and high temperature (30/24 and 32/22 ºC) treatments in Exp. 1 and 2, during grain filling, respectively

could be because of fermentation losses during the ensiling process. Ensiling is a microbial fermentation process and the microbes use sugars and other easily fermentable carbohydrates to produce volatile fatty acids and lactic acid. Also CO_2 is formed, which is associated with a loss of fermentable carbohydrates. Therefore the silage fermentation process should be as fast as possible. The longer the period of ensiling process is, the more undesired fermentation takes place and the less fermentable carbohydrates remain for fermentation by rumen micro-organisms (Cone *et al*., 2008b). Besides these, other nutritional losses could be caused by respiration of the plants in the initial phase of ensiling, as long as oxygen is present (McGechan, 1990). Losses continue to occur until all oxygen is used and a low pH is reached, causing a stable phase in the ensiling process (Pahlow *et al.*, 2003).

Rumen gas production of maize silage reduced as the ensiled duration increased (up to 16 weeks) in our experiments showing in an inverse relationship between the two. Cone *et al.* (2008b) found no influence of ensiling period (up to 26 weeks) on net gas production and its rate. Use of different silos and sample types could be the reasons of the discrepancy between the results. Cone *et al.* (2008b) used freeze dried, whereas we used fresh silage samples.

Another reason of an inverse relationship between gas production and ensiling period could be a prolonged viability of *Lactobacillus buchneri* during ensiling (up to a 52 weeks) even at a low pH under anaerobic conditions (Kleinschmit and Kung, 2006). Moreover prolonged ensiling sometimes results in silages with lower concentrations of lactic acid, but higher acetic acid when compared with the same silage that had been ensiled for only a few months (Kleinschmit and Kung, 2006; Herrmann *et al.*, 2011). Indeed, some strains of LABs are capable of using lactic acid anaerobically when glucose is limiting (Der Bedrosian *et al.*, 2012). Furthermore, *Lactobacillus buchneri* is metabolically viable in silages for months, even at a low pH, and thus can reduce the feeding value of maize silages (Der Bedrosian *et al.*, 2012) .

Conclusions

The findings of this thesis show an overview of how characteristics of rumen starch degradation are influenced by genotype, environmental conditions and their interactions. This also covers the impact of different handling processes of starch, like oven-drying. Moreover, it also includes how the feeding quality and value of maize silage is influenced by ensiling temperature and duration. The following conclusions can be drawn based on the results presented in this thesis.

Rumen starch degradation is influenced by maize genotypes

- Rumen starch degradation was strongly influenced by starch content, structure and composition along with composition of endosperm i.e. vitreousness (Chapter 3).
- Rumen starch degradation was negatively related with amylose content and vitreousness of the endosperm, whereas it was positively related to amylopectin content (Chapter 3).
- Lower rumen starch degradation values in genotypes with higher amylose contents could be because only amylase can be crystalline (Chapter 3).
- The negative relationship of rumen starch degradation with vitreous endospermic (or rumen escape starch) genotype could be due to less accessibility of starch, in a complex starch-zein matrix, to degrading enzymes (Chapter 3).

Rumen starch degradation is influenced by growth temperature during grain filling

- There was a significant effect of growth temperature on starch degradation of maize kernels through its influences on dry matter accumulation rate and duration (Chapters 2 and 3).
- Less ruminal starch was degraded under unfavourable lower (due to imbalanced grain filling rate and duration, i.e. slower dry matter / starch accumulation rate) and higher growth temperatures (faster dry matter /

starch accumulation rate but reduced grain filling duration) at the same harvest date (Chapter 3).

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Rumen starch degradation is influenced by stage of maturity

- Harvesting stage determines the moisture content of the maize kernels and hence plays an important role in the feeding values of maize kernels (Chapters 2 and 3).
- The influence of harvesting stage on rumen degradation is related to specific dry matter and starch contents. The feeding value of maize kernels shows an optimum at specific water content (Chapters 2 and 3).
- The reduced rumen starch degradation of maize kernels could also be due to an increased vitreousness at the latest maturity stage (Chapter 3).

Rumen starch degradation is influenced by post-harvesting processing like oven-drying

- Rumen starch degradation is a microbial fermentation process, and enzymes can only be active when water is present. Rumen starch degradation was significantly influenced by the process of oven-drying (Chapter 4).
- Evaluating the feeding value of starch in maize kernels after oven drying will provide an underestimation of the true value (Chapter 4).
- High amylose, due to its crystalline nature, showed a relatively large difference between fresh and oven-dried values of rumen starch degradation (Chapter 4).

Maize silage feeding value is influenced by ensiling conditions and duration

- Ensiling temperature and duration played an important role in the rumen degradation of maize silage through its influence on pH of the maize silage (Chapter 5).

Future prospects for rumen starch and maize silage degradation

The following suggestions can be made, based on the results of the present studies, to accurate measure the feeding value of maize:

- To develop a feed evaluation model that takes account of genotype (starch structure, composition, type of endosperm), its growing conditions, especially temperature during the grain filling period and the maturity stage. Also different processes of starch, like oven-drying, should be taken into account.
- Breeders, especially maize breeders, need to keep paying attention towards the influence physical stress, when breeding for maize, as environmental conditions significantly influence the feeding value of maize.
- Present feed evaluation systems use dried kernel data to evaluate the feeds, but this present study suggests that fresh kernels produce significantly higher values of gas production and ruminal starch degradation. Moreover the breeders also use NIRS data of oven-dried kernels in breeding programmes, which may result in mistakes. So, feed evaluation systems and breeders should also take into account the difference between fresh and oven-dried data to predict the exact feeding value of maize.

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Summary

Grain filling, starch degradation and feeding value of maize for ruminants

Maize (*Zea mays* L.) has three main possible uses: as food, as feed for livestock and as raw material for industry (mainly bioethanol production). However 65% of the global maize production is used for animal feed. Maize is extremely diverse in its genetic make-up. This also relates to concentration and structure (i.e. amylose or amylopectin) of starch and composition (i.e. amylose: amylopectin) and type of endosperm of the kernel. Maize kernels consist of ~61% of starch, which plays an important role in the quality as animal feed. There is a need to understand the rumen starch degradation of maize and how it is influenced by different factors, such as genotype, environmental conditions during crop growth and managerial factors or factors associated with sample processing. The rate and extent of starch degradability can be highly variable. This variability can be due to diversity in starch structure and composition and also in type of endosperm among maize genotypes. Consequently, the genotypes can differ in feeding value. The research described in this thesis has been conducted to understand and evaluate the effects of genotype of maize and environmental factors (temperature during grain filling) on the chemical composition and nutritive value of the maize kernel. Stage of maturity is of special interest for farmers and maturity stage, in terms of dry matter concentration, can play an important role in the feeding value of maize. So, there is also a need to evaluate the effect of the maturity stage on the rumen starch degradation of maize kernels. In addition, maize is fed to cows as fresh silage; the physical structure and chemical composition are changed after oven-drying. Therefore, it is important to study oven-drying effects on the feeding value of maize kernels. Oven-drying can affect rumen starch degradation possibly due to its effect on physical structure and molecular conformation of starch. Maize is also used as silage across the world and its feeding quality also depends on the ensiling conditions (one of them is temperature) and duration. Therefore, the influence of ensiling temperature and duration on activities of microorganisms, and, ultimately, on its feeding quality was investigated.

This thesis is an attempt to understand how rumen *in vitro* starch degradation of maize kernels is influenced by genotype, environment and harvest date. Moreover, it also helps to understand how processes like oven-drying influence

rumen *in vitro* starch degradation. In addition, this thesis is a step to evaluate the effects of ensiling temperature and duration on the feeding quality of maize silage for lactating cows.

Maize experiments were conducted with diverse sets of maize genotypes, varying in starch structure and composition and in type of endosperm. Crops were grown under controlled conditions in a greenhouse or on different sowing times and different soils in the field. This gave a wide source of multiple variations in which genotype and environment played a major role (Chapter 2). The maize kernels were harvested at different stages of grain filling to further evaluate the optimum maturity stage for their feeding quality in lactating cows. Maize kernels were analysed for moisture concentration, water content and kernel weight. The different growth temperatures during grain filling significantly influenced the rate of dry matter accumulation, its duration and final kernel weight in the different maize genotypes. Sub-optimal growth temperatures reduced grain filling rate but increased its duration, whereas supra-optimal temperatures increased the grain filling rate but reduced its duration and also the grain filling rate when expressed in thermal time. Hence both sub- and supra-optimal growth temperatures resulted in a reduced yield and kernel weight. The higher kernel dry weight and kernel yield were obtained when the day/night temperatures during grain filling were (24/18 and 27/17 °C) as these temperatures resulted in a combination of high grain filling rate and long duration of grain filling at the same time. Final kernel yield per kernel or m^2 was also significantly influenced by genotype, soil type and sowing time. Waxy and vitreous genotypes showed higher final kernel weight and final kernel yield than other genotypes included in the studies. Water contents were also influenced by growth temperature, genotype and sowing time. Highest maximum water contents were observed for lower growth temperature (18/12 and 22/12 °C) and for the dent genotype in the greenhouse experiments. Highest maximum values were recorded for non-vitreous genotypes and early sowing in the field experiments. The intermediate temperature (24/18 and 27/17 °C) and dent genotype took longest to achieve maximum water contents in the glasshouse conditions. In the field experiments, high values of thermal time to reach the maximum water contents were

recorded for the clay soil, vitreous genotypes and intermediate sowing time. In this thesis, a bi-disciplinary approach was chosen, combining crop physiology and animal nutrition. The gas production technique was used to analyse the diverse and huge sources of variations for degradability, especially starch degradability, of maize kernels at different maturity stages (Chapter 3). The parameters characterizing the degradability of maize were described and were used to identify factors influencing the degradation of maize kernel starch per unit of starch were described. The relationship between stage of maturity and degradation of maize was identified based on pollination date. Maize kernel samples (collected from experiments described in Chapter 2) were analysed for their starch, dry matter and ash concentrations. Then *in vitro* gas production technique was used to evaluate the feeding value of the maize kernels in rumen fluid from lactating cows. The starch degradation was calculated at various incubation times (6, 12 and 20 h) from observed values of gas production and starch concentrations by using an already established equation. Finally, starch degradation was calculated per gram of starch. Rumen starch degradation was significantly influenced by the genotype of the maize, varying in starch structure, composition and type of endosperm. The rumen starch degradation was positively related to the amylopectin content of the genotypes and was negatively related to the amylose content and vitreousness of the endosperm. The rumen starch degradation was also influenced by the growth temperature during the grain filling period, as it affected the rate and duration of the starch biosynthesis. The optimum growth temperature (24/18 and 27/17 °C), resulting in high dry matter contents (Chapter 2), also resulted in high rumen starch degradation. Maturity also influenced the rumen starch degradation. Rumen starch degradation decreased with advancing maturity, most probably because of increasing vitreousness. We assume changes in zein concentration (not measured) to have played a role. Vitreous endosperms show a lower susceptibility of the starch to amylase, either originating from microorganisms or from the ruminant. Moreover, advancing maturity results in a higher dry matter concentration, influencing the rumen starch degradation negatively.

As ruminants eat maize as a fresh, ensiled product, there was a need to test the impact of oven-drying on the feeding value of maize kernels. Fresh maize kernels were stored at -18 °C, whereas oven-dried (70 °C) kernels were stored at room temperature until further analysis. Samples were also analysed for their dry matter, ash and starch concentrations. The gas production technique was used to investigate the difference between fresh and oven-dried kernels in terms of feeding value (Chapter 4). The results showed that oven-drying significantly reduced the gas production and hence the feeding value of maize kernels. This was observed across different sources of variation in genotypes, material grown under different environmental conditions and harvested at different maturity stages. Oven-drying dehydrates the maize kernels, whereas ruminal microbes need well hydrated kernels to start their activities. This could be one of the reasons why oven-drying significantly reduced rumen starch degradation. More importantly, a significant interaction between genotype and oven-drying was also found. The high-amylose genotypes were more affected by oven-drying than all other genotypes. Only amylose can become crystalline by retro-gradation, which can occur during oven-drying. Crystallization negatively affects the rumen starch degradation.

A series of experiments with different ensiling temperatures and duration of ensiling (from non-ensiled to up to 16 weeks) was conducted. Silage samples were ensiled at three different temperatures and were removed at specific periods and were then stored a -18 °C (Chapter 5). Maize silage samples were evaluated in rumen fluid of lactating cows using the gas production technique. The results showed that the ensiling temperature affected the decline rate of the pH and the final pH of the maize silages. The ensiling temperature influenced pH decline per ensiled day, possibly by influencing microbial activities. Based on the results, maize silage needs an optimum ensiling temperature (12 °C) for better feeding value. Lower or higher than the optimum (12 °C) resulted in slower decline of the pH and a higher final pH. Moreover, it reduced the maize feeding value. It was found that not only the final pH, but also the decline rate of the pH was important for the feeding quality of the maize silages. The 12 °C treatment which resulted in a faster decline in pH also resulted in a higher gas production, hence feeding values. It was observed that

non-ensiled material showed higher gas production values than ensiled maize at all temperatures. The results indicated that as the ensiling duration increased, the gas production decreased. This could be due to the prolonged activity of lactic acid producing bacteria, which could have remained viable for longer periods (up to 16 weeks). This resulted in reduced rumen degradation of maize silage with prolonged ensiling (up to 16 weeks) duration.

Finally, there is comprehensive discussion (Chapter 6) about the main findings of all the experimental chapters presented in this thesis. It summarizes and evaluates how maize genotypes, their growing environment and their genotype × environment interactions influence the rumen starch degradation. Moreover, the discussion also contains how different post-harvest handling processes of maize like oven-drying affect rumen starch degradation of maize kernels. This chapter also discusses how important maturity stage is, along with aforementioned factors, for the feeding value of maize kernels. Maturity stage is, perhaps, considered most important from farmers' perspective. Maize silage feeding value as influenced by ensiling conditions (temperature) and duration is also discussed in the same chapter. The same chapter (Chapter 6), finally, also highlights future prospects of maize feeding quality from plant breeding, crop physiology, animal nutrition modelling and feed evaluation systems' perspectives.

Samenvatting

Grain filling, starch degradation and feeding value of maize for ruminants

Maïs (*Zea mays* L.) wordt vooral voor drie doeleinden geteeld: voedsel, veevoer en grondstof voor andere industrieën (voornamelijk ten behoeve van de productie van bio-ethanol). Van de wereldwijde productie van maïs wordt 65% gebruikt voor diervoeder. Maïs kent een grote genetische diversiteit. Dit geldt ook voor de concentratie en de chemische structuur (bijvoorbeeld amylose of amylopectine) van het zetmeel, maar ook ten aanzien van zetmeelsamenstelling (bijvoorbeeld de verhouding amylose: amylopectine) en het type endosperm in de korrel. Maïskorrels bestaan voor ongeveer 61% uit zetmeel en dit zetmeel is mede bepalend voor de kwaliteit van maïs als diervoeder. Er is behoefte om de afbraak van zetmeel van maïs in de pens beter te begrijpen en meer inzicht te verkrijgen in de invloed van verschillende factoren op die zetmeelafbraak. Belangrijke beïnvloedende factoren kunnen zijn genotype, omgevingsomstandigheden tijdens gewasgroei, teelttechniek en factoren die samenhangen met de verwerking van de monsters. De snelheid en mate van afbraak van zetmeel kunnen sterk uiteenlopen. Deze variabiliteit kan het gevolg zijn van genetische diversiteit in zetmeelstructuur en samenstelling, maar ook van verschillen in type endosperm. Bijgevolg kunnen genotypen verschillen in voederwaarde. Het in dit proefschrift beschreven onderzoek werd uitgevoerd om de effecten van het genotype van maïs en van omgevingsfactoren (temperatuur tijdens de korrelvulling) op de chemische samenstelling en de voederwaarde van de maïskorrel te karakteriseren en te evalueren. Het rijpheidsstadium is van bijzonder belang voor de boeren en het rijpheidsstadium, uitgedrukt in percentage droge stof, kan een belangrijke rol spelen in de voederwaarde van maïs. Dus is er ook een noodzaak om het effect van het rijpheidsstadium op de zetmeelafbraak van maïskorrels in de pens te evalueren. Bovendien wordt maïs als vers kuilvoer aan koeien gevoerd, terwijl de fysieke structuur en chemische samenstelling tijdens het drogen van de monsters in een oven veranderen. Daarom is het van belang om ook na te gaan wat het drogen in een oven doet met de voederwaarde van de maïskorrels. Drogen in een oven kan de afbraak van zetmeel in de pens mogelijk beïnvloeden via effecten op de fysische structuur en de moleculaire conformatie van het zetmeel. Maïs wordt ook in de hele wereld gebruikt als kuilvoer en daarom hangt de voederwaarde ook af van de omstandigheden tijdens het inkuilen (bijvoorbeeld

temperatuur) en van de bewaarduur van de kuil. Daarom werd de invloed van de temperatuur tijdens het inkuilproces en van de bewaarduur van de kuil op de activiteiten van de micro-organismen, en uiteindelijk op de voederwaarde in dit proefschrift onderzocht.

In dit proefschrift is beschreven hoe de in-vitro-afbraak van zetmeel van maïskorrels in de pens wordt beïnvloed door genotype, milieuomstandigheden en oogstdatum. Daarnaast draagt het proefschrift bij aan het begrijpen van de invloed van het drogen in een oven op de in-vitro-afbraak van zetmeel. Bovendien zet dit proefschrift een eerste stap om de effecten van temperatuur en de duur van het inkuilen op de voederwaarde van snijmaïs voor lacterende koeien te begrijpen.

Experimenten werden uitgevoerd met verschillende sets van maïsgenotypen, variërend in structuur en samenstelling van het zetmeel en in de aard van het endosperm. Gewassen werden geteeld onder gecontroleerde omstandigheden in een kas of in het veld met verschillende zaaitijden en verschillende bodems. Gezamenlijk leverde dit een grote en diverse bron van variatie in zetmeelkwaliteit op, waarbij genotype en milieu een belangrijke rol speelden (Hoofdstuk 2). De maïskorrels werden geoogst in verschillende stadia van korrelvulling om na te gaan wat het beste rijpheidsstadium is ten aanzien van de voederwaarde voor lacterende koeien. Maïskorrels werden geanalyseerd op vochtgehalte, hoeveelheid water per korrel en korrelgewicht. De verschillende groeitemperaturen tijdens de korrelvulling bleken de snelheid van drogestofophoping, de duur ervan en het uiteindelijke korrelgewicht van de verschillende maïsgenotypen significant te beïnvloeden. Suboptimale groei-temperaturen verlaagden de korrelvullingssnelheid maar verhoogden de korrelvullingsduur, terwijl supra-optimale temperaturen de korrelvullingssnelheid verhoogden, maar de korrelvullingsduur verkortten en ook de korrelvullingssnelheid uitgedrukt in thermotijd verlaagden. Vandaar dat zowel sub- als supra-optimale groeitemperaturen resulteerden in een verminderde opbrengst en korrelgewicht. Hogere droge stof gewichten van de korrel en droge stof opbrengsten werden verkregen als de dag/nacht-temperaturen tijdens de korrelvulling 24/18 of 27/17 °C bedroegen, omdat deze temperaturen tegelijkertijd resulteerden in een combinatie

van een hoge korrelvullingssnelheid en een lange korrelvullingsduur. De uiteindelijke opbrengst per korrel of per vierkante meter werd ook significant beïnvloed door genotype, grondsoort en zaaitijd. De waxy en vitreuze genotypen vertoonden hogere uiteindelijke korrelgewichten en uiteindelijke korrelopbrengsten dan de andere genotypen die bij het onderzoek betrokken waren. De hoeveelheid water in de korrel werd ook beïnvloed door de groeitemperatuur, genotype en zaaitijd. In de kasproeven werden de hoogste waarden voor de maximale hoeveelheid water in de korrels gevonden bij de lagere groeitemperaturen (18/12 en 22/12 °C) en voor het dent type. In de veldproven werden de hoogste waarden voor de maximale hoeveelheid water in de korrels gevonden bij de niet-vitreuze genotypen en de vroege zaai. In de kasproeven duurde het bij de tussenliggende temperaturen (24/18 en 27/17 °C) en het dent type het langst om de maximale hoeveelheden water in de korrel te bereiken. In het veldproeven werden de hoogste waarden van thermotijd om de maximale hoeveelheden water in de korrel te bereiken waargenomen voor kleigrond, de vitreuze genotypen en de middelste zaaitijd.

In dit proefschrift, zijn twee disciplines, te weten de gewasfysiologie en de diervoeding, gecombineerd bij de aanpak. De gasproductietechniek werd gebruikt om de diverse, grote bronnen van variatie betreffende de afbreekbaarheid, in het bijzonder van zetmeel, van maïskorrels van verschillende rijpheidsstadia te analyseren (Hoofdstuk 3). Parameters die de afbreekbaarheid van maïs karakteriseren zijn beschreven en hiervan is gebruik gemaakt om factoren die de zetmeelafbraak van de maïskorrel per eenheid zetmeel bepalen, te identificeren. De relatie tussen het rijpheidsstadium en de afbraak van maïs werd vastgesteld op basis van de datum van bestuiving. Monsters van maïskorrels (verzameld in de proeven beschreven in Hoofdstuk 2) werden geanalyseerd op hun gehalten aan zetmeel, droge stof en as. De gasproductietechniek werd vervolgens gebruikt om de voederwaarde van de maïskorrels in pensvloeistof van lacterende koeien te evalueren. De zetmeelafbraak werd op verschillende incubatietijdstippen (na 6, 12 en 20 uur) berekend met behulp van een reeds gepubliceerde vergelijking en gemeten waarden van gasproductie en zetmeelgehalten. De zetmeelafbraak werd

berekend per gram zetmeel. De afbraak van zetmeel in de pens werd significant beïnvloed door het genotype van de maïs, omdat de gebruikte genotypen varieerden in zetmeelstructuur, zetmeelsamenstelling en het soort endosperm. De zetmeelafbraak in de pens was positief gerelateerd aan het amylopectinegehalte van de genotypen en was negatief gerelateerd aan het amylosegehalte en hoe vitreus het endosperm was. De zetmeelafbraak in de pens werd ook beïnvloed door de groeitemperatuur tijdens de korrelvulling, vanwege het effect op de snelheid en de duur van de zetmeelsynthese. De optimale groeitemperatuur (24/18 en 27/17 °C), die resulteerde in een hoog drogestofgehalte (Hoofdstuk 2), leidde ook tot een hoge zetmeelafbraak in de pens. Het rijpheidsstadium beïnvloedde ook de zetmeelafbraak in de pens. De zetmeelafbraak in de pens daalde naarmate de rijpheid verder gevorderd was, hoogstwaarschijnlijk vanwege een toenemende vitreusiteit. We nemen aan dat veranderingen in het zeïnegehalte (niet gemeten) eveneens een rol hebben gespeeld. Vitreus endosperm heeft een lagere gevoeligheid van het zetmeel voor amylase, afkomstig van micro-organismen of van de herkauwer. Bovendien resulteert een verder gevorderde rijpheid in een hoger drogestofgehalte, hetgeen de zetmeelafbraak in de pens negatief beïnvloedt.

Omdat herkauwers maïs eten als een vers, ingekuilde product, en niet na droging in een oven, was het wenselijk om de gevolgen van drogen in een oven op de voederwaarde van maïskorrels te testen. Verse maïskorrels werden opgeslagen bij -18 °C, terwijl in een oven (bij 70 °C) gedroogde korrels bij kamertemperatuur werden bewaard voor verdere analyse. Monsters werden geanalyseerd op hun gehalten aan droge stof, as en zetmeel. De gasproductietechniek werd toegepast om het verschil tussen verse en in een oven gedroogde korrels qua voedingswaarde te onderzoeken (Hoofdstuk 4). De resultaten toonden aan dat het drogen in een oven de gasproductie (en dus de voederwaarde van maïskorrels) significant verlaagde. Dit verschil werd waargenomen ongeacht de bron van variatie in kwaliteit, of het nu genotype was, of milieuomstandigheden tijdens de opkweek van het plantmateriaal of het oogsttijdstip. Het drogen in een oven lijdt tot verlies van vocht in de maïskorrels, terwijl microben in de pens goed gehydrateerde korrels nodig hebben om hun activiteiten te kunnen starten. Dit kan een van de redenen zijn waarom

drogen in een oven leidt tot een aanzienlijk lagere zetmeelafbraak in de pens. Nog belangrijker was het feit dat we een significante interactie vonden tussen genotype en het effect van drogen. De genotypen met een hoog gehalte aan amylose werden meer beïnvloed door het drogen in een oven dan alle andere genotypen. Slechts amylose kan een kristallijne vorm aannemen door retrogradatie, hetgeen kan optreden bij het drogen in een oven. Kristallisatie heeft negatieve gevolgen voor de zetmeelafbraak in de pens.

Een reeks experimenten werd uitgevoerd met verschillende inkuiltemperaturen en bewaarduur van de kuilen (van niet-ingekuild materiaal tot materiaal dat maximaal 16 weken als silage werd bewaard). Silagemonsters werden ingekuild bij drie verschillende temperaturen en werden op specifieke tijdstippen verzameld en opgeslagen bij -18 °C (Hoofdstuk 5). Snijmaïsmonsters werden in pensvloeistof van lacterende koeien geëvalueerd met behulp van de gasproductietechniek. De resultaten toonden aan dat de inkuiltemperatuur invloed had op de snelheid waarmee de pH van de kuil daalde alsmede op de uiteindelijke waarde van de pH van de maïskuil. De inkuiltemperatuur beïnvloedde de pH-daling per ingekuilde dag, waarschijnlijk door het beïnvloeden van de activiteit van de microben. Op basis van de resultaten kan geconcludeerd worden dat snijmaïs de beste voederwaarde levert bij een optimale inkuiltemperatuur van 12 °C. Een lagere of hogere temperatuur dan 12 °C resulteerde in een tragere afname van de pH, een hogere uiteindelijke waarde van de pH en een lagere voederwaarde van de maïs. Niet alleen de uiteindelijke pH, maar ook snelheid waarmee de pH daalde bleek belangrijk te zijn voor de voederwaarde van de maïskuilen. Snijmaïs ingekuild bij 12 °C vertoonde een snellere daling van de pH, maar ook een hogere gasproductie, en dus een betere voederwaarde. Het materiaal dat in zijn geheel niet werd ingekuild bleek een hogere gasproductie te vertonen dan ingekuilde maïs, ongeacht de inkuiltemperatuur. De resultaten gaven aan dat naarmate de kuil langer werd bewaard de gasproductie daalde. Dit kan te wijten zijn aan de langdurige activiteit van melkzuur producerende bacteriën, die kennelijk gedurende een langere periode (tot 16 weken) levensvatbaar kunnen blijven. Dit resulteerde in een verminderde pensafbraak van snijmaïs bij langdurige bewaring van de kuilen (tot 16 weken).

Tenslotte zijn de belangrijkste bevindingen van alle experimentele hoofdstukken in dit proefschrift uitgebreid bediscussieerd (Hoofdstuk 6). Het afsluitende hoofdstuk evalueert hoe maïsgenotypen, hun groeiomstandigheden en de genotype × omgeving interacties de zetmeelafbraak in de pens beïnvloeden. Bovendien bespreken we hoe de processen gedurende de verschillende behandelingen na de oogst van maïs (zoals het drogen in een oven) de zetmeelafbraak van maïskorrels in de pens beïnvloeden. Dit hoofdstuk bespreekt ook hoe belangrijk rijpheid is, samen met de eerder genoemde factoren, voor de voederwaarde van de maïskorrels. Rijpheid is misschien wel de belangrijkste factor vanuit het perspectief van de boer. De voederwaarde van snijmaïs zoals die wordt beïnvloed door inkuilcondities (temperatuur) en bewaarduur wordt ook besproken in hetzelfde hoofdstuk. Tenslotte duidt Hoofdstuk 6 de toekomstperspectieven voor een betere voederwaarde van maïs vanuit het oogpunt van de plantenveredeling, de gewasfysiologie, het modelleren van veevoedingsaspecten en voederwaarderingssystemen.

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Mazhar Ali

List of publications

Journal publications

Khan, N.A., J.W. Cone, W.F. Pellikaan, M.A. Khan, P.C. Struik, W.H. Hendriks. Changes in fatty acid content and composition in silage maize during grain filling. J. Sci. Food Agric., 91 (2011), pp. 1041–1049.

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Response of maize genotypes contrasting in starch type to temperature during grain filling. Submitted to Journal of Agronomy and Crop Science.

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Starch degradation in rumen fluid as influenced by genotype, climatic conditions and maturity stage of maize grown under controlled conditions. Submitted to Animal Feed Science and Technology.

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Oven-drying reduces ruminal starch degradation in maize kernels. Submitted to Animal Feed Science and Technology.

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Ruminal degradation of maize silage is influenced by temperature and duration of ensiling. Submitted to Journal of Animal Physiology and Animal Nutrition.

Poster Presentations

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Feeding value of maize as influenced by growth temperature and maturity stage in maize kernels.

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Feeding value of maize as influenced by soil types and maturity stage in maize kernels.

Oral Presentation

Ali, M., J.W. Cone, W.H. Hendriks, P.C. Struik. Effect of amylose and amylopectin content on rumen starch degradation of maize kernels at two maturity stages.

PE&RC and WIAS PhD Training and Education Statement

With the training and education activities listed below the candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) and the Wageningen Institute of Animal Sciences (WIAS).

Review of literature (6 ECTS)

- Rumen starch degradability of maize kernels as influenced by growing conditions genotypes, maturity and post-harvest processing

Writing of project proposal (4.5 ECTS)

- A new approach to evaluate the feeding value of starch in silage maize to enhance future breeding and feed evaluation

Post-graduate courses (4.5 ECTS)

- Long term dynamics of food and human development; PE&RC (2008)
- Statistics of life science; WIAS (2008)

Competence strengthening / skills courses (4.5 ECTS)

- Information literacy, including Endnote; WGS (2008)
- Ethics and philosophy of animal science; WGS (2008)
- Scientific writing; WGS (2009)
- Presentation skill; WGS (2009)
- Effective behaviour in your professional surroundings; WGS (2009)
- Techniques for writing and presenting a scientific paper; WGS (2011)
- Interdisciplinary research: crucial knowledge and skills; WGS (2011)
- Imaging science: video and audio in scientific communication; PE&RC (2012)
- Data management; WGS (2013)

PE&RC and WIAS Certificate

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- WIAS Introduction course; WIAS (2008)
- PE&RC Days (2011-2013)

Discussion groups / local seminars / other scientific meetings (5 ECTS)

- WIAS Science days; poster presentation (2008-2012)
- Mamo Animal Nutrition Group; lectures / seminars / weekly (2008-2013)

International symposia, workshops and conferences (4.1 ECTS)

- ISNH8 Conference; Aberystwyth University, Wales, UK (2011)
- International Livestock Nutrition Conference; oral and poster presentation; Pakistan (2013)

Mazhar Ali was born on 6th of June 1980 in Jampur, District Rajanpur, Punjab, Pakistan. His father's name is Muhammad Hussain Khan. After completing higher secondary school education, he started to study BSc (Agri-agronomy) at College of Agriculture, Dera Ghazi Khan, sub-campus University of Agriculture, Faisalabad (UAF). He did a Master in Agri-agronomy (high first division) in 2005 from the UAF. He worked three months as Temporary Technical Sales Officer (TTSO) in Syngenta, Pakistan .He resigned from this job to start a PhD.

In early 2007, he qualified for a Higher Education Commission (HEC) of Pakistan's scholarship to follow a programme overseas. Later in the same year, he started his PhD at Wageningen University, The Netherlands. The PhD project he worked on was a bi-disciplinary project of the "Centre for Crop Systems Analysis" and the "Animal Nutrition group". He worked six years on this project under the supervision of a highly qualified team of supervisors; Prof. Dr. Ir. Paul C. Struik, Prof. Dr. Ir. Wouter H. Hendriks and Dr. John W. Cone. The main objective of his research was to evaluate the different factors, either intrinsic or extrinsic, influencing the rumen degradation of maize kernels and silage. The intrinsic factors included genotypes differing in starch structure, composition and endosperm and in their earliness. Extrinsic included growing conditions, different maturity stages, and post-harvest processing (oven-drying) for maize kernels. Another objective was to study the influence of ensiling conditions (temperature) and duration on feeding value of maize silage.

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