Selection and characterization of maize (*Zea mays* L.) for Methionine and Tryptophan content

by

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"The plant breeder is an explorer into the infinite. He will have 'no time to make money,' and his castle, the brain, must be clear and alert in throwing aside fossil ideas and rapidly replacing them with living, throbbing thought followed by action. Then, and not until then, shall he create marvels of beauty and value in new expressions of materialized force, for everything of value must be produced by the intelligent application of the forces of Nature which are always awaiting our commands".

-- Luther Burbank, Fundamental Principles of Plant Breeding, 1902

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Chapter 1: GENERAL INTRODUCTION

1. Introduction

Because maize is a major food and feed crop in many parts of the world, it is important to optimize its nutritional potential. However, it is well established that the maize protein is nutritionally limited by deficiencies in the amino acids Lysine, Methionine, and Tryptophan. When used as a feed, maize is supplemented with soybean meal and synthetic amino acids to complement the maize proteins and provide a balanced diet to the animal. This is a costly process in meat production systems that can be reduced with a nutritionally enhanced maize grain. In order to develop maize germplasm with improved content of limiting amino acids, a breeder needs to be knowledgeable of the genetic variability for the levels of amino acids, of the genetic effects controlling the levels of amino acids in order to design a breeding plan for the improvement of germplasm for the amino acid content.

The major goal of this research is to design a breeding method that is effective for the selection of Tryptophan and Methionine specifically and to test the nutritional quality of the improved populations. The potential of the recurrent selection method was tested with two random-mated maize populations. Another goal is to characterize commercial maize germplasm for its Tryptophan and Methionine content and to determine the genetic effects, such as general combining ability, specific combining ability, reciprocal effects and maternal effects associated with Tryptophan and Methionine levels in maize seeds.

2. Literature review

2.1. Essential amino acids

The monogastric animals, or simple non-ruminants, require specific dietary essential amino acids. By definition, amino acids are deemed essential if an organism does not synthesize them and they must therefore be supplied in the diet. If one of the essential amino acids is limiting, the deficiency results in a negative nitrogen balance (Berg, 2002). Non-essential amino acids are not required *per se* in the diet but are required metabolically for protein synthesis (Cheeke, 1999). In addition, a non-essential amino acid may be the limiting factor in growth if its level in the diet is insufficient and if the essential amino acids responsible for its synthesis are similarly only present in marginal amounts (Wiseman, 1987).

In animal diets, the efficiency of protein utilization is dependent upon two types of factors: external factors, which relate to rearing conditions and internal factors, relating directly with the protein itself (Berg, 2002). The nutritional value associated with a protein may be estimated by comparing the ingested nitrogen to that which is actually retained for protein synthesis. Requirements for a specific monogastric animal depend on its metabolic peculiarities and are dependent on the genotype, the performance, the method of feeding, and the environment in which the animal is reared (Wiseman, 1987). Also, the quality protein requirement is different for growth than for maintenance and is affected by sex and by species (Cheeke, 1999). According to Wiseman (1987), all factors that introduce variability in performance of individual animals within a population will increase requirements. Signs of protein deficiency include anorexia, reduced growth rate, reduced or negative nitrogen balance, reduced efficiency of feed utilization, etc. Specific lesions may appear with

deficiencies for certain amino acids: Tryptophan deficiency produces eye cataracts; Methionine deficiency produces fatty liver (Pond, 1995).

2.2. Amino acid levels in maize

What determines amino acid levels

Osborne (1924) reported the classification of the seed proteins into four classes based on solubility. These classes are the albumin (water-soluble), the globulin (salt-soluble), the prolamin (aqueous alcohol-soluble), and the glutelin (not soluble in water, saline solutions, or aqueous alcohol). The seed storage proteins, which include the globulins in legume seeds and the prolamins in cereal seeds, are the most abundant proteins. In maize, the prolamins are called zeins. Because of their abundance, they are the primary determinants of the amino acid composition in maize kernels (Larkins et al., 1993). Osborne and Clapp (1908) characterized the composition of the zein proteins and reported that zeins lack of two essential amino acids, Lysine and Tryptophan.

Genes involved in determining amino acid balance

Several mutant genes affect the amino acid balance in maize. The most studied mutant genes are *Opaque-2* and *Floury-2*. Both *opaque-2* and *floury-2* genes have been described as early as 1935, though their nutritional potential was not revealed until the 1960's (Mumm, 1935; Singleton and Jones, 1935). As will be discussed later, the high Lysine content of the *opaque-2* endosperm mutant discovered by Mertz *et al* (1964) revolutionized the status of protein quality research. In addition to the discovery of the exceptional levels of Lysine in *opaque-2* mutant, the *floury-2* mutant has an altered amino acid balance with high

concentrations of Lysine and Methionine (Nelson et al., 1965). Another gene, *Dzr1* (*e.g. Zpr10/22*), regulates Methionine levels by controlling the level of the δ-class zeins (Benner et al., 1989).

2.3. Studies on quality traits in maize

Traditional breeding for quality traits

i. Selection for protein quantity

A number of maize breeders utilized traditional breeding methods to increase the protein quantity in maize kernels. In 1896 Hopkins initiated a selection program for protein and oil content that averred to be the most prominent study in this area of research (Hopkins, 1899). This program became known as the Illinois long-term selection experiment. These experiments consisted of repeated cycles of selection for oil and protein and show that mass selection was successful in changing kernel composition in maize. Significant genetic variability in the maize populations was maintained after 65 generations of selection (Dudley and Lambert, 1974). From Iowa State University, Frey *et al* (1949) reported that selecting for an increase in total protein in the maize kernel results in an increase in the zein fraction in the endosperm proteins. Given that the zein fraction has poor nutritional quality due to its lack of Tryptophan and Lysine, it seems likely that selection for protein content will result in lower protein quality. In 1951, Frey concluded from his study on the interrelationships of proteins and amino acids in corn that the protein in selections for low protein was more nutritionally balanced than the protein in selections for high protein. Thus, in order to

improve protein quality by selecting for protein quantity, it would be best to select for low protein (Frey, 1951).

ii. Selection for protein quality

Zuber and Helm (1972) studied the improvement of protein quality, defined as an increase in the Lysine content of open-pollinated varieties, without the utilization of endosperm mutants. They used a recurrent selection method as a means of improving the amino acid balance. They were able to increase the level of Lysine from one cycle of selection to the next, though the mean protein values remained essentially the same. They also suggest that different environmental conditions could have caused such changes as the two cycles were grown in different seasons (Zuber and Helm, 1972).

Mutation breeding and QPM

The findings of Mertz, Bates and Nelson in 1964 are well known regarding the *opaque-2* mutant. The mutation yields a lower content of the zein proteins in the endosperm. It also provides 69% more Lysine than that in normal maize seeds (Mertz et al., 1964). After extensive work and great hope for the use of *opaque-2* maize to overcome the problem of malnutrition in certain developing countries, the problems related to the *opaque-2* phenotype became quite obvious. The major problems that were identified by early 1970 were reduced grain yield, soft and chalky kernel phenotype, greater vulnerability to ear rot, greater moisture content which conflicted with the dry-down of the seed, and lower rate of germination (Vasal, 2001). Efforts to improve the *opaque-2* phenotype were initiated at CIMMYT in the mid-1970s. The combination of two mutants, *sugary-2* and *opaque-2*, prompted much interests and hopes. The characteristics of this combination were slightly

better than *opaque-2* maize in terms of kernel hardness and ear rot tolerance but were not improved in terms of grain yield and germination rate. However, the protein quality of the double-mutant combination was sometimes better than that of the *opaque-2* maize (Mertz, 1992).

In the 1980's, CIMMYT was engaged in developing quality protein maize (QPM) by combining the *opaque-2* gene with genetic modifiers that improved the hardness of the maize kernel. Eventually, the scientists at CIMMYT were able to develop QPM material that yielded as well as their normal counterparts (Vasal, 2001). *Opaque-2* populations were established and scientists have anticipated that the accumulation of modifiers and favorable genes would be an effective tool for improving traits related to seed quality (Lorenzoni and Motto, 1985).

Biotechnology approaches to improving protein quality

With the advent of genetic engineering, a number of studies have proven the feasibility of improving the Met content in a variety of crops. Lai and Messing (2002) constructed a transgene based on a chimeric *Dzs10* gene by replacing the 3' UTR with a transcript of the cauliflower mosaic virus that would enhance the level of expression in maize endosperm cells. The level of Methionine was increased as a result of the accumulation of the Dzs10 protein, a high Methionine zein. As milk protein has the potential to provide good nutritional enhancement with its excellent amino acid profile, Yang *et al* (2002) sought to synthesize a porcine α-lactalbumin gene construct. Expression of this synthetic gene in maize kernels resulted in a 20% increase in Lysine levels (Bicar et al., Unpublished). Transformation of narrow-leafed lupin (*Lupinus angustifolius* L.) seeds expressing the

sunflower seed albumin (SSA) gene resulted in a 94% increase in Methionine when compared to the wild type (Molvig et al., 1997). Molvig *et al* (1997) reported that not only was the protein quality improved in the transgenic seeds, but also the true protein digestibility, the biological value, and the net protein utilization. In tobacco, a group of researchers created and transformed a chimeric gene encoding a Brazil nut Methionine-rich seed protein (Altenbach et al., 1989). The accumulation of the protein in the tobacco seeds resulted in a 30% increase in the levels of Methionine.

Biotechnological approaches in improving the nutritional quality of crops may be promising, though both advantages and disadvantages have to be elucidated. Benefits of such technology are that genes expressing protein from a different organism than the target host can have beneficial nutritional attributes. In the study conducted by Yang *et al* (2002), a porcine milk protein with good digestibility, bioavailability, and amino acid balance was introduced into maize. Disadvantages of using biotechnological tools are the difficulties associated with plant transformation and expression of foreign proteins, and the potential introduction of allergenic properties. Several studies have reported such difficulties. Molvig *et al* (1997) reported that molecular approaches in improving the amino acid balance were hindered by the difficult regeneration of grain legumes and by the unstable expression of the modified protein in the target host. The Methionine-rich Brazil nut protein expressed in soybean revealed to have allergenic properties (Nordlee et al., 1996). The transgenic soybean varieties were not commercialized (Lai and Messing, 2002). When transferring and expressing a heterologous protein into the seeds of a target plant, the protein may undergo degradation. If the protein does not accumulate, it would not be expected to have an impact on the amino acid balance of the transgenic plant (Altenbach et al., 1989).

2.4. Testing the nutritional quality of improved germplasm

Improved germplasm for nutritional quality through the use of biotechnology or the use of mutants has been tested with animal feeding trials. Upon the discovery of the increased Lysine and Tryptophan contents in the *opaque-2* mutant, Mertz *et al* conducted an animal body weight experiment to compare *opaque-2* maize and a normal hybrid, Indiana 453. They showed a 3.6-fold increase in rat weight gain when the rats were fed *opaque-2* maize compared to the rats fed Indiana hybrid 453 (Mertz, 1965). For the transgenic lupin seeds with the increased Methionine content, rats gained more weight than the control group when the lupin seeds were their sole nitrogen source (Molvig et al., 1997). Also, Lai and Messing (2002) reported an increased weight gain in two-day old chicks fed Methionine-enhanced diets. Another report on testing Methionine-enhanced maize in a feeding trial using one-day old chicks shows similar food utilization by the chickens on the control diet and those on the enhanced diet. A significant difference was shown in the growth performance of the young chicks (Messing and Fisher, 1991).

2.5. Methods for quantifying Trp & Met in maize kernels

In any plant breeding program aimed at improving the content of amino acids, it is critical to have a method for quantifying the amino acids of interest accurately and inexpensively. Three types of methods are normally used to quantify the target amino acids: bioassay, chemical assay, or chromatography. Shankman *et al* (1943) used strains of *Lactobacillus arabinosus*, auxotrophic for specific amino acids, to determine the concentrations of eight amino acids. The content of the amino acids was based on the

amount of lactic acid produced by the bacteria. In 1995, Wright and Orman proposed another microbiological method for the analysis of Methionine in maize and soybean seeds. They used the bacteria *Pediococcus cervisiae*, auxotrophic for Methionine, and measured the turbidity, a representation of bacterial growth, as an indication of the Methionine content in the sample. Though this method may not provide the best analytical accuracy, it provides high throughput that would appeal to plant breeders (Wright and Orman, 1995). Hernandez and Bates (1969) determined that microbial assays and chromatographic techniques used in the determination of Tryptophan were expensive, tedious, and time consuming. They developed a chemical method, using iron chloride to characterize Papain-hydrolyzed protein in terms of Tryptophan content. This method was used extensively in the maize breeding program at CIMMYT. In 1985, Sastry and Tummuru proposed a different method for analyzing the protein hydrolysates for Tryptophan. After alkali hydrolysis of the sample, this method takes advantage of the colored product of the reaction between Tryptophan, thioglycolic acid and sucrose under acid conditions to measure Tryptophan levels spectrophotometrically. This method is highly sensitive, rapid and simple (Sastry and Tummuru, 1985). The American Organization of Analytical Chemists recognizes ion exchange chromatographic methods for the determination of both Tryptophan (1995a) and Methionine (1995b).

3. Thesis organization

This thesis consists of the general introduction, two papers, a general conclusion, references cited and acknowledgements. The first paper is entitled "Nutritional improvement of maize populations by recurrent selection for amino acid levels". This study shows not

only the feasibility of improving maize populations for their Methionine and Tryptophan levels through traditional breeding methods but also the potential of establishing a divergent selection program for those specific amino acids. In addition, the nutritional quality of populations resulting from divergent selection for Methionine and Tryptophan levels was evaluated by chemical analysis and with a rat feeding trial. The second paper, "Variability and genetics effects for Methionine and Tryptophan in commercial maize germplasm," shows the existing Methionine and Tryptophan variability among genetic groups in a maize breeding germplasm and the different genetic effects associated with those traits. A general discussion including the general conclusions and insights for the improvement of amino acid levels in maize by traditional means follows the second paper. References for each paper and for the general introduction section are cited at the end of each chapter.

All of the papers were written by the primary author with the guidance and assistance of Dr. Scott for the biochemical analyses and the basis of the research projects and Dr. Lamkey for the statistical analyses. The two papers are multi-author papers. Dr. Stahly, from the Department of Animal Science at Iowa State University, helped conduct the feeding trial (Chapter 2). Rich Clayton, Research Associate for Dr. Stahly, helped with the daily chores of the trial. Christian Buffard and Bill Forgey of Pau Seeds, Inc, a subsidiary company of Bayer Crop Science, contributed to the experimental design of Chapter 3. Philippe Chartier and Bill Forgey, maize breeders at Pau Seeds Inc, generated all the maize that was used in the study (Chapter 3). All chemical analyses for Methionine and Tryptophan content were conducted by the primary author in Dr. Scott's lab with technical support for grinding seeds and weighing samples. All data analysis was conducted by the primary author.

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Chapter 2: NUTRITIONAL IMPROVEMENT OF MAIZE POPULATIONS BY RECURRENT SELECTION FOR AMINO ACID LEVELS

A manuscript to be submitted for publication in the journal *Maydica / Crop Science*

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1. Abstract

Some essential amino acids in maize are nutritionally limiting for animal diets. Plant breeding offers the means to improve the nutritional quality of maize. One breeding method that could be effective for this purpose is recurrent selection. The objective is to improve a population while conserving genetic variation within the population. The potential of this method to improve maize populations for their content in two amino acids, Methionine (Met) and Tryptophan (Trp), was tested. The objectives of this study are (1) to determine the effect of selection for the amino acid composition in maize, (2) to develop populations diverging in both Trp and Met levels, and (3) to determine the effect of selection on the nutritional value of the populations. Two random-mated maize populations, BS11 and BS31, were chosen for this study on the basis of their protein content and variability. The two populations were treated independently and selections were categorized as high Met, low Met, high Trp, and low Trp within each population. One cycle of half-sib family divergent selection for Met and Trp was completed. For the Cycle 1 BS11 populations, the values for the high Met and Trp selections were significantly higher than the values for the low Met and Trp selections. These data suggest that a divergent selection program is a useful method for producing

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populations with improved Met and Trp content. Entries from the BS11 populations that differed significantly in Met content were evaluated in a rat feeding trial. While there were no statistical differences in weight gain or in feed efficiency, the rats fed on the Low Met diet consumed statistically more feed than the rats on the High Met diet. The divergent populations developed in this study will be a valuable resource for researchers interested in the genetics and biochemistry of kernel amino acid content.

2. Introduction

Maize is often a major component of animal diets; therefore modifications that improve its nutritional value are desirable. In meat production, the greatest expense is the cost of feed. Dietary improvements that result in decreased feed consumption per weight gain would therefore reduce the cost of meat production. In monogastric diets, maize is nutritionally limited by deficiencies in Lysine, Methionine, and Tryptophan. These deficiencies can be corrected by supplementation, although this adds to the cost of the diet. Genetic improvements resulting in increased levels of these amino acids would be valuable because they would reduce the amount of supplementation required.

Genetic improvement of crop amino acid content has a long history. Traditional breeding methods were first utilized when scientists began exploring the composition of the maize kernels. In 1899, Hopkins reported the improvement in the chemical composition of the corn kernel through selection (Hopkins, 1899). A decade later, Osborne and Clapp (1908) characterized the composition of the zein proteins and reported that zeins lack of two essential amino acids, Lysine and Tryptophan. The combination of those studies led scientists to devote their efforts to the improvement of maize protein quality through

breeding (Dudley et al., 1974; Frey, 1951; Frey et al., 1949; Zuber and Helm, 1972). In the 1960's, the focus of breeding for protein quality shifted to mutation breeding when Mertz *et al* (1964) discovered that the *opaque-2* mutant increased the Lysine content in the maize endosperm. The poor agronomic characteristics such as reduced yields, soft endosperm, ear rot susceptibility, and poor germination of these mutants with improved amino acid balance reduced the practical value of mutant varieties. In the mid-1970's, researchers at the Center for the Improvement of Maize and Wheat (CIMMYT) in Mexico began research in improving the agronomic traits of *opaque*-2 maize. They combined the *opaque*-2 gene with genetic modifiers that improved the hardness of the maize kernel. The resulting improved maize germplasm is referred to as quality protein maize, or QPM (Mertz, 1992). Clearly, plant breeding is an effective method for making genetic improvements to crops.

Recurrent selection is a cyclical breeding procedure. The objective of recurrent selection is to change the mean value of a quantitative trait in a population using selection, while maintaining genetic variability. In completing one cycle of recurrent selection, individuals from a population are evaluated and superior individuals are selected. These individuals are intermated to generate progeny that constitute the population in the next cycle of selection. Recurrent selection procedures have been used for plant, ear, and kernel traits with a great deal of success (Hallauer, 1992). Recurrent selection programs are generally planned on a long-term basis. For example, the Illinois long-term experiment for oil and protein content has been underway for 100 generations (Dudley and Lambert, 2002). In this experiment, selection for higher and lower oil and protein content in maize was effective (Dudley and Lambert, 1974). In recurrent selection, the effectiveness of selection depends on the

heritability of the trait of interest, which in turn depends on the complexity of the trait, or the number of genes involved in the expression of the trait of interest (Hallauer, 1992).

In breeding programs, evaluation of the success of the program is a key component. In studies involving nutritional improvement of a crop, a feeding trial is commonly conducted to test the resulting improved crop. The laboratory rat (*Rattus norvegicus*) is a good experimental model for research on nutrition because of its moderate size, high reproductive performance, adaptability to diverse diets, and tractable nature. Inbred strains are generally used to limit the experimental error due to genotype differences. The dietary requirements of rats are well established and are reviewed in Nutrient Requirements of Laboratory Rats (Nutrient requirements of laboratory rats, 1995).

The objectives of this study were (1) to determine the effect of selection for amino acid composition in maize, (2) to develop populations diverging in Trp levels and populations diverging in Met levels, and (3) to determine the effect of selection on the nutritional value of the populations.

3. Materials and methods

3.1. Populations used in this study

Two different maize populations identified to use in this study. One population was derived from BS11, a population originally designated as "Pioneer Two-ear Composite". It was developed by crossing southern prolific material and Corn Belt lines (Hallauer, 1967). The second population was derived from the BS31 population, another open-pollinated synthetic population derived from FS8A(T)C4 (Lamkey, 2002). The FS8A population was initially developed at the Florida Agricultural Experiment Stations and released in 1988.

Germplasm from Southeastern U.S., Corn Belt, and Tropical sources, respectively, account for approximately 30, 22, and 48% of FS8A(T). The initial objective in the development of this population consisted of intermating a wide range of accessions selected for resistance to southern corn leaf blight (Horner, 1990). The BS11 and BS31 material used in this study has been under selection for agronomic performance for multiple cycles of recurrent selection.

3.2. Breeding strategy

One hundred and two hundred half-sib ears from the populations BS11 and BS31, respectively, were produced in the summer of 2000. The Met and Trp content of five randomly selected kernels from each ear was determined, analyzed, and the ears were categorized based on the results. The five highest and five lowest ears for each amino acid were selected from each population, giving eight categories, each containing five selected ears. These categories were called BS11HT, BS11LT, BS11HM, BS11LM, BS31HT, BS31LT, BS31HM, and BS31LM. Thus, the BS11HT category represents the ears from the BS11 population with the highest content of Trp (HT), while BS31LM represents the ears from BS31 with the lowest content of Met (LM), and so on.

In the summer of 2001, seed from each selected ear, thereafter referred to as selection, was planted in two adjacent rows at the Iowa State University Agronomy Farm located in Boone, Iowa giving a plot containing 80 rows. The plants *within* each selection were intermated in a chain-sib mating design. The resulting ten to twelve ears per row were harvested individually and five randomly selected kernels from each ear were analyzed for content of Trp and Met.

In the summer of 2002, two experiments were planted at the Iowa State University Agronomy Farm in Boone, Iowa. In the first experiment, hereafter called the Inter-mate experiment, intermating was carried out *among* the selections made in 2000 in the following manner. For each category (i.e.BS31HT), a balanced bulk was made with seed from each of the five selections in the category. The eight resulting bulks were then planted in five adjacent rows of 25 kernels. The plants in each bulk were intermated in a chain-sib mating design. About 50 ears were produced in each category. These ears were harvested individually and the Trp and Met content of five randomly selected kernels from each ear was determined. In each category, five of the approximately 50 ears were selected on the basis of their amino acid content as before. These selections constitute the Cycle 1 population in each category.

The second experiment, thereafter called the Intra-mate experiment, was similar to the first experiment except intermating was carried out *within* the selections. Upon analysis of the material generated in the summer of 2001, five ears were selected from each category as either the highest or the lowest ears depending on the category. Three adjacent rows of 25 kernels were planted in 2002 for each selection, giving 40 three-row plots. The plants were intermated *within* each three-row plot in a chain-sib mating design. Approximately 30 ears from each three-row plot were harvested in a bulk. The Trp and Met content of five randomly selected kernels from each bulk was analyzed as before, and three the best three bulks were selected to represent the HT, LT, HM, and LM categories of each population. A sub-sample of the bulks derived from BS11 representing each category (i.e. BS11LT) was sent to the University of Missouri-Columbia Experiment Station Chemical Laboratories for a complete amino acid analysis by ion exchange chromatography according to the AOAC

official method 982.30 E (a,b,c). Also, NIR spectroscopy was used to predict the protein, oil and starch content of each bulk.

Comparisons among treatment means for the different categories within the populations and among the two diets were determined using an F-test implemented by the GLM procedure of SAS (SAS-Institute, 2000).

3.3. Preparation of samples for analysis of Met and Trp levels

From each experiment, each ear of maize was shelled and packaged individually. From each ear, five randomly selected whole kernels were ground using a Wiley Mill with a 40-mesh screen. Each ground sample was stored in an Eppendorf tube. With the flap of the tube open, the samples were then dried for four hours at 65°F, after which the tubes were closed and stored in ambient conditions. Samples were analyzed in 96-well plates using a Randomized Complete Block Design including two checks (B101 and B45o2), and six standards consisting of known concentrations of commercially prepared amino acids. The B101 inbred was chosen as a check because it has high levels of Methionine (Hallauer and Wright, 1995). The inbred B45o2 inbred was used as a check for high Tryptophan. Commercially-prepared, pure amino acids were used as standards in concentrations of 5, 20, 35, 60, 75, 100 μM for Methionine and 0, 100, 240, 300, 480, 600 μM for Tryptophan. The analysis was replicated on three plates with a different randomization of the entries on each plate (i.e. three blocks). The checks were replicated twice within a plate and the standards three times within a plate. Ten milligrams of each ground sample and check were weighed into the well of a V-bottom, 96-well microtiter plate.

3.4. Protein hydrolysis

Each sample was subjected to enzymatic hydrolysis using Pepsin. To each well, 200 μL of 0.1 mg/mL Pepsin solution in a KCl-HCl pH2 buffer was added. The plate was then sealed, covered with a lid, and placed in a 37 °C shaking incubator for approximately 15 hours. After the incubation period, the plate was centrifuged at 3000 rpm for 20 minutes, after which the supernatant was removed for further analysis.

3.5. Assay for Trp

The method for the determination of Tryptophan in maize kernels is a modified version of the one originally described by Sastry and Tummuru (1985). Twenty microliters of hydrolysate or standard was transferred directly into the wells of a flat-bottom, 96-well assay plate. The plates were sealed between operations to prevent evaporation. For each plate, the assay solution consisted of 9.5 mL of concentrated HCl, 250 μL 2.5% Thioglycolic Acid (TGA), and 250 μL 10% sucrose. This solution was prepared and warmed to 42°C for 23 minutes to allow the solution to turn yellow. Under a fume hood, 80 μL of this assay solution was added to the hydrolysate in the assay plate. The plate was then shaken on a plate shaker for three minutes, after which the optical density at 510 nm was immediately determined with a microplate reader.

3.6. Assay for Met

The microbiological method for the determination of Methionine in maize kernels is similar to that described by Wright and Orman (1995). An auxotrophic strain of *Escherichia coli*, P4x (Jacob and Wollman, 1961), was used in this assay. The inoculum was prepared in

M9 media supplemented with 10 μL of 1 mg/mL Methionine solution per 5 mL M9 media (Maniatis, 1982) and grown to late log phase. Ten microliters of hydrolysate or a standard was transferred directly into a flat-bottom, 96-well assay plate. The plates were sealed between operations to prevent evaporation. To each well, 100 μL of M9 media and 2 μL of the inoculum were added. The plate was then sealed, covered with a lid, and placed in a 37 °C shaking incubator for seven hours. After the incubation period, the plates were placed on a plate shaker for 3 minutes and the 595 nm light scattered by the sample was determined using a microplate reader.

3.7. Protocol for rodent feeding trial

The selections made for high and low Methionine in the BS11 populations from the Intra-mate experiment were used for the feeding trial because those selections had the greatest difference between the High and the Low categories. The remnant grain of the bulks selected for the feeding trial was ground using a pin mill located at the Center for Crop Utilization Research at Iowa State University. The maize kernels were ground to an average particle size of 90 US Standard. Forty one-month-old female Sprague-Dawley rats were penned individually in wire-floored cages to facilitate the daily collection of any spilled feed, and were acclimated to these conditions for three days. During this time, the rats were fed standard corn, which was supplemented with the necessary minerals and vitamins, and water *ad libitum*. On the fourth day, the 30 rats were weighed and 15 rats of similar weights were randomly allotted to one of two dietary treatments: BS11HM or BS11LM. Again, feed and water were provided *ad libitum*. In addition to the dietary treatments, the basal diet consisted of minerals, vitamins, soybean meal concentrate, and supplements of the essential amino

acids Lysine, Threonine, and Tryptophan (Table 4). Feed intake and body weight gain were measured at four-day intervals until each rat achieved 170 ± 6 grams body weight.

4. Results

4.1. Trp and Met concentrations in starting populations

In order to determine the feasibility of direct selection for Trp and Met content, two populations, BS11 and BS31 that are under investigation for their agronomic traits, were chosen on the basis of their protein content and variability. One-hundred and two-hundred individuals from the respective populations were analyzed for their Trp and Met content. Figures 1 and 2 show the distributions of Trp and Met content in these individuals. Selections were made within these two populations to generate high and low subpopulations from each starting population. In the BS11 population, the mean Trp content of the selections in the High category was 145% higher than the mean of the selections in the Low category. Similarly for the Met content, the mean of the High category was 137% higher than the mean of the Low selections. The distribution of the starting BS31 population is given relative to the mean of the population for each trait. There was more variation for Trp (relative values of ±2.40 from the mean) than for Met (relative values of –0.77 for the Low tail and +0.95 for the High tail). These selections formed eight new populations, four from BS11 and four from BS31, that were selected either for high or low Trp or Met levels.

4.2. Effect of selection on random-mated populations

Two experiments were carried out using the eight populations generated above. In the Inter-mate experiment, we completed one full cycle of recurrent selection by intermating

among the selections within each population in the summer of 2002. This allowed us to evaluate the potential of recurrent selection for changing amino acid levels. Approximately forty ears resulting from intermating among the selections within each population were analyzed for their Trp and Met content. The mean Trp and Met values for each category are reported in Table 1 under the Inter-mate heading. Statistically significant differences in Met and Trp levels were observed between the high and low populations derived from BS11. For both Trp and Met levels, the High category was found significantly higher than the Low category. The mean Met content of BS11HM was 7.15% higher than the mean of BS11LM. Similarly, the mean of BS11HT showed a 6.37% increase when compared to the mean of BS11LT. The Trp and Met populations derived from BS31 did not have statistically significant differences in Met and Trp levels. However, the mean Trp content of BS31HT was 2.39% higher than the mean of BS31LT, and the mean Met content of BS31HM was 1.23% higher than the mean of BS31LM.

In the summer of 2001, the Intra-mate experiment was initiated to observe the Trp and Met levels in the progeny of the selections made in the starting populations. Ultimately, it was served to provide grain for a feeding trial by increasing the seed and the selection pressure for Met and Trp content. In this experiment, selections were planted and intermating was carried out *within* each selection in each category. The Trp and Met content of the ears resulting from each selection were then analyzed, and the mean Trp and Met values for each category are reported in Table 1 under the Intra-mate heading. In BS11, the mean Trp content of the populations derived from high and low Trp selections were not significantly different at the $p=0.05$ level. However, the mean Met content of the population derived from HM was significantly higher than that for LM. In BS31, the mean Trp content

of the population derived from LT selections was significantly higher than the mean Trp content of the population derived from HT selections at the p=0.05 level. Also, the mean Met content of the population derived from LM selections was significantly lower than the mean Met content of the population derived from HM selections at the p=0.05 level. Given these differences in amino acid levels, we decided to test the nutritional value of this material in a rat feeding trial. In order to generate the approximately 10 kg of grain required for the trial, it was necessary to grow the selections for another generation.

Thus, in the summer of 2002, with the seed resulting from mating *within* each selection in each population, we conducted another round of selection for grain to be used in a feeding trial. As in 2001, selections were planted in rows and intermated within each row. Ears from each row were combined to produce a bulk of grain representing each selection within each category. Bulks representing each of the five selections in each of the eight categories (BS11HT, BS11LT, BS11HM, BS11LM, BS31HT, BS31LT, BS31HM, BS31LM) were analyzed in triplicate for their Trp and Met content. The results of this analysis are plotted by selection category as a function of their Trp content and their Met content (Figures 7 and 8). The checks B101 and B45o2 are also included in the graphs as a reference. The differences between the means of the High and the Low categories of BS31 were statistically significant ($\alpha = 0.05$) for Met only, with a 16% difference between the means. Though not statistically different, the mean of the HT category was higher than the mean of the LT category. The differences between the means of the High and the Low categories of BS11 were the greatest, and were statistically significant ($\alpha = 0.05$), being 5.3% for Trp and 23% for Met.

4.3. Analysis of the chemical composition of the selections resulting from intermating *within* selections for each population

With the greatest and significant differences between the means of the High and the Low categories of BS11, we then decided to analyze the four categories from BS11 in greater detail in order to design an appropriate feeding trial. Three bulks from each category were selected based on their high or low Trp or Met content and then bulked by category to form four batches: BS11HT, BS11LT, BS11HM, and BS11LM. To confirm the effectiveness of the selection protocol and to further characterize the selections, the complete amino acid composition of these batches was determined (Table 2). This complete amino acid composition provides additional information regarding the effect of selection for a specific amino acid on the other amino acids. The HM grain is substantially higher in Met content than the LM grain, establishing that the selection based on our Methionine assay was effective. We could not draw a similar conclusion about selection for Trp because the complete amino acid analysis did not have enough precision to detect the difference we measured with our assays. Figure 9 illustrates the differences between the HM and the LM selections and the HT and the LT selections for all amino acids as well as the total protein content calculated by adding the individual amino acid levels. A negative differential implies the amino acid either decreased when selecting for HM or HT or increased when selecting for LM or LT. Overall, there was an increase in total protein content and in most other amino acids when selecting for HM or HT. A notable exception is that the Lysine content decreased when selecting for HT. The Valine and Isoleucine content also decreased when selecting for HT. When selecting for HT and for HM, there was a distinct increase in Glutamic Acid.

To further characterize this material, NIR spectroscopy was used to predict the total protein, oil, and starch content of each class (Table 3). Only starch was found to be significantly different between the High and the Low categories for both Trp and Met (Table 4). No significant differences were observed between the High and the Low category for neither oil nor protein. Though not significant, the High categories for both amino acids had a higher mean protein content than the Low categories. In addition, the Low categories for both amino acids had a significantly higher mean starch content than the High categories.

4.4. Rat feeding trial of high and low Met BS11 selections

Having established that significant differences exist in amino acid composition between the HM and LM grain from BS11, we next compared these entries in a rat feeding trial to determine if the measured changes resulted in an altered nutritional quality. The grain was supplemented to formulate two diets that were not limited in Lysine, Threonine or Tryptophan as summarized in Table 5. Approximately 7.5 kilograms of each diet were prepared for the feeding trial and stored at 4°C until use. Rats were weighed on the first day of the trial and at four-day intervals thereafter. The average weight of the rats on each diet and for each four-day interval is shown in Figure 10. There was no statistical difference between the weight gain of the rats fed on High Met (HM) and of the rats fed on Low Met (LM). Daily feed consumption was monitored by subtracting the mass of spilled feed from the mass of the feed provided. Figure 11 presents the averaged feed consumption for the rats at four-day intervals. Overall, the feed consumption for the rats fed on the LM diet was statistically higher (α=0.05) than that for the rats fed on the HM diet. From these data, the feed efficiency, as measured by the body weight gain per amount of net feed ingested, was

calculated (Figure 12). For the first 16 days of the trial, there was no statistical difference in feed efficiency between the rats on the HM diet and those on the LM diet. Between the dates of 8 April and 12 April, the feed efficiency diverged with the rats on the HM diet being more efficient than the rats of the LM diet. This divergence was not statistically significant. Table 6 summarizes the growth performance of the female rats for the feeding trial.

5. Discussion

5.1. Effect of selection

In order to investigate the effect of selection for Trp and Met content in maize populations, two experiments were conducted. The first one, which was referred to as the Inter-mate experiment, was essentially the completion of one cycle of recurrent selection. The second experiment, which was referred to as the Intra-mate experiment, was designed to further diverge the Trp and Met levels in progenies derived from pre-selected parents while to increasing the seed for a feeding trial. In general, the data from both experiments suggest that a divergence in Trp and Met levels by selection is possible. Results from the Intra-mate experiment show that for populations derived from BS11 and BS31, the mean Met content of the High category was significantly higher than the mean Met content of the Low category. The response to selection for differing Trp levels in the two populations was not as effective as the response to selection for Met. In the BS11 population, there were no statistical differences between the mean Trp value of the High category and that of the Low category. In the BS31 population, the mean Trp value for the Low category was significantly higher than the mean Trp value for the High category.

The data from the Inter-mate experiment confirm the feasibility of the divergent selection for Trp and Met levels. In general, the response to selection for Met was similar to the response in the Intra-mate experiment but the response to the selection for Trp was small (BS11) or negligible (BS31). This response to selection may suggest that Trp is a more complex trait than Met, thus making it difficult to select for. Another explanation is the analytical method utilized for the determination of Trp may not be accurately determining the Trp content of the maize proteins. In BS11, the variation in Met content is similar to the variation in Trp content, while in BS31, there is more variation in Trp content than in Met content.

Direct selection for a specific amino acid has an effect on the complete amino acid profile. When selecting for HM or HT, the total protein increased and the content of most amino acids increased as well. However, it was shown that selecting for HT resulted in a decrease in the Lysine content. This contradicts the widely-held notion that Lysine and Tryptophan concentrations are correlated (Hernandez and Bates, 1969). As Lysine is the first amino acid to be limiting in maize diets, this decrease may require additional Lysine supplements to be provided to animal feed. Other amino acids that clearly decreased when selecting for HT are Valine and Isoleucine, two other essential amino acids. For these reasons, special attention should focus on the complete amino acid profile when selecting for a specific amino acid, as another amino acid may become limiting following the selection process. Further investigation may include the correlation between the limiting amino acid and other amino acids that are only slightly more abundant. In regards to the protein content, selecting for higher levels of either Trp or Met substantially increased the protein content.

These results are different than those of Zuber and Helm (1972) in which they were able to increase the Lysine levels in maize kernels without significantly altering the protein content.

The data from the Inter-mate experiment suggest and confirm that a recurrent selection program is a feasible method to establish a divergent selection for amino acid levels in maize. Significant differences were found between the Low and the High categories of the BS11 population only. The fact that the response to selection from the two populations differed may be explained by two reasons: genetic drift and inbreeding depression (Keeratinijakal and Lamkey, 1993; Smith, 1983). Genetic drift is a result of the small effective population size. It is possible that genetic drift in the starting BS31 population may have affected the frequency of favorable alleles for high Trp levels. Again, the analytical methods for the determination of Trp concentration may need to be modified for more accurate measurements. The success of a breeding program using the recurrent selection method is to maintain genetic variability from one cycle to the next. Further cycles of selection are necessary to corroborate the positive results of the first cycle of recurrent selection.

5.2. Rats fed HM corn consume less feed than rats fed LM corn with no difference in gain

In order to determine if selection for Methionine content increased the nutritional value of the populations under selection, we conducted a rat feeding trial in which the effects of the High and Low populations on rate of body weight gain, feed efficiency and feed consumption were compared. Rats fed the diet based on the High Met population consumed significantly less feed that those on the Low Met diet, with no difference in body weight gain or feed efficiency. In other feeding trials conducted with monogastric animals, the response

to supplemental Met has been inconsistent (Russell et al., 1986). In only one of seven treatments, feed efficiency of growing pigs fed maize-soybean meal diets was improved when Met was the only amino acid added to the diet (Russell et al., 1986). In chickens fed a low protein diet of soybean and herring meal, it was reported that the feed consumption was reduced and that the feed efficiency was not affected when compared to the control (Fockedey and Arnould, 1978). The inconsistent effects of Met supplementation may explain the results of our feeding trial with female rats.

A decrease in feed consumption such as we observed would normally be accompanied by a change in body weight gain or feed efficiency, and we did not observe either of these responses. One possible explanation for this is that the diets had different moisture contents. We therefore analyzed the moisture content of the diets and found them to be not significantly different (data not shown).

Females generally reach their optimum growth at approximately 140 grams before their reproductive stage begins. We can anticipate male rats would be more suitable in such a feeding trial as they gain more weight for a longer period of time. Possibly, this would result in a detectable growth performance with the male rats fed on the two diets.

The experimental maize diets differed not only in Met concentrations but also in other amino acids and in the total protein content. As those differences did not suffice to make substantial differences in the growth performance of the rats, further investigation of the complete diet (corn meal and supplements) is necessary to characterize the energy content of the diet and other factors that may be limiting in the diet. The total nitrogen content of the complete diets was higher for the HM diet (2.491) than for the LM diet (2.3585). Thus, the total protein available in the diets was not altered with the supplementation of the soybean

meal. The protein content in the HM diet remained higher than the protein content in the LM diet, regardless of the other sources of protein. The percent moisture was not significantly different between the diets. Discrepancies due to the moisture content in the diets were not a factor. From these observations, we can conclude that the rats fed on the LM diet consumed more feed to meet the dietary demands for protein than the rats fed on the HM diet.

6. Figures and Tables

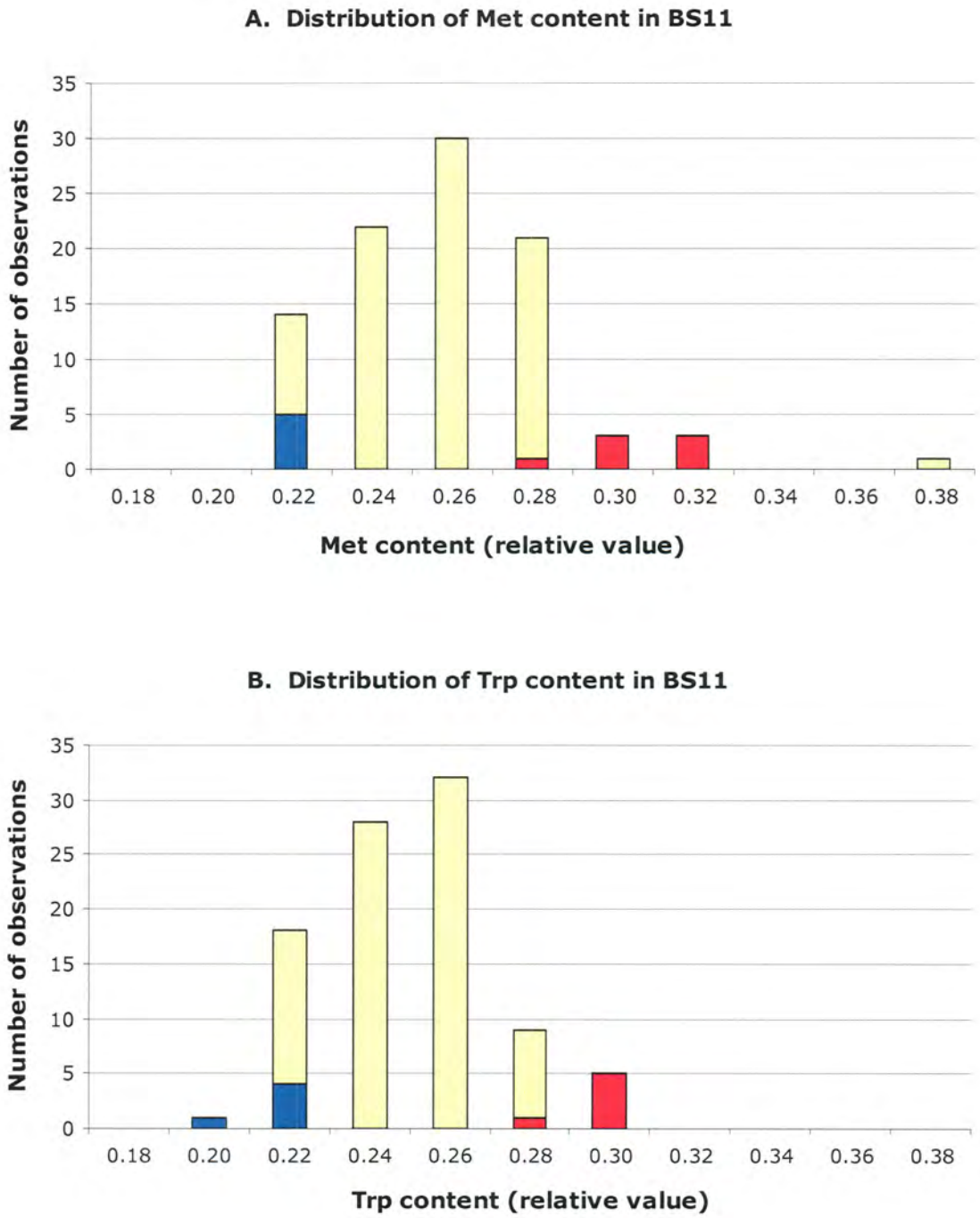

Figure 1. Distribution of the initial BS11 population for its Met content (A) and Trp content (B). The levels of Met and Trp represent the optical density measurement corrected for the mass of the sample. In blue are the selections of the Low categories (BS11LM and BS11LT) made for Cycle 1 of the recurrent selection program. In red are those for the High categories (BS11HM and BS11HT). The overall population mean for its Met value was 0.25 and 0.24 for its Trp value.

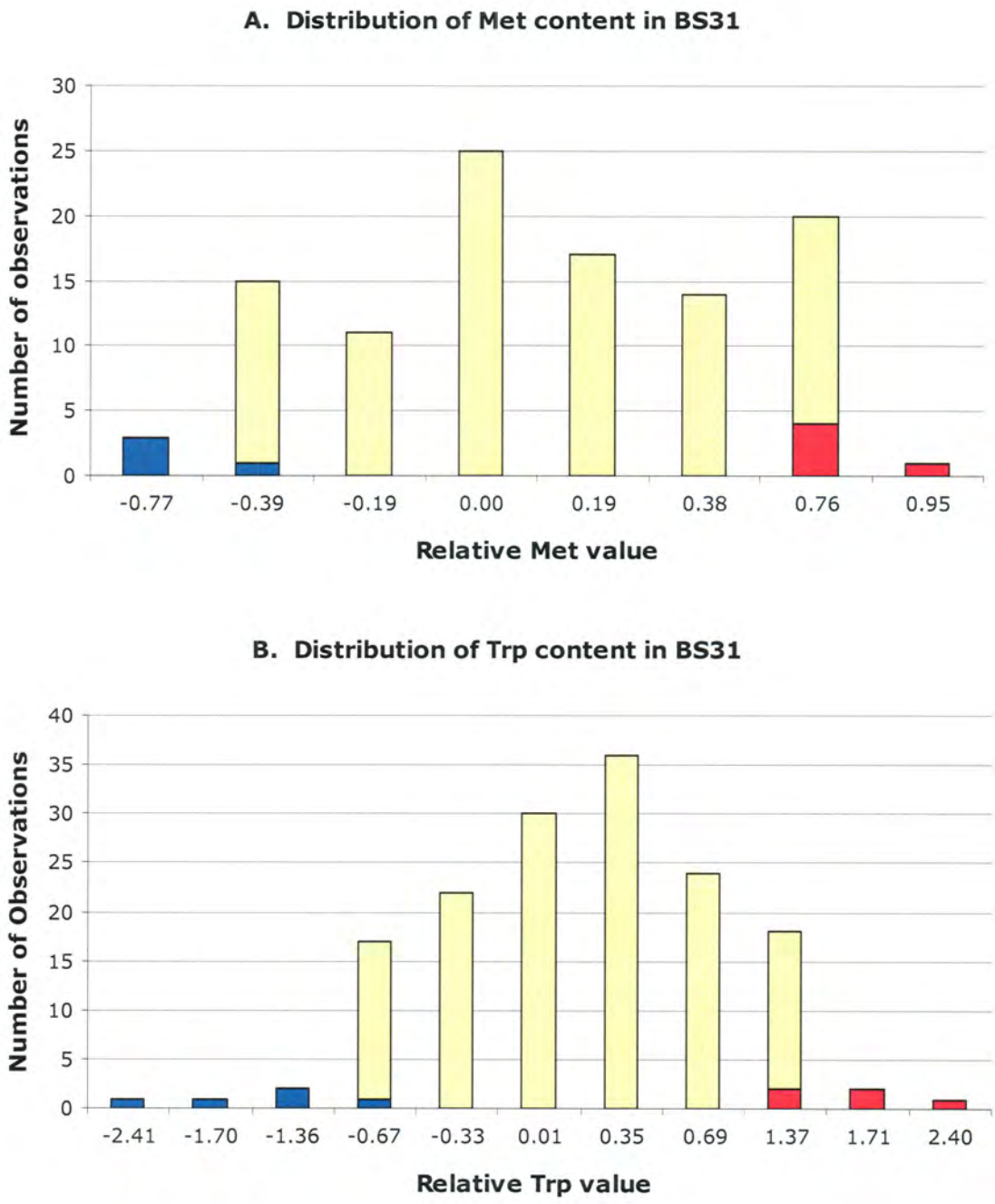

Figure 2. Distribution of the initial BS31 population for its Met content (A) and Trp content (B). The Met and Trp values are relative to the overall mean value for the population. In blue are the selections of the Low categories (BS31LM and BS31LT) made for Cycle 1 of the recurrent selection program. In red are those for the High categories (BS31HM and BS31HT).

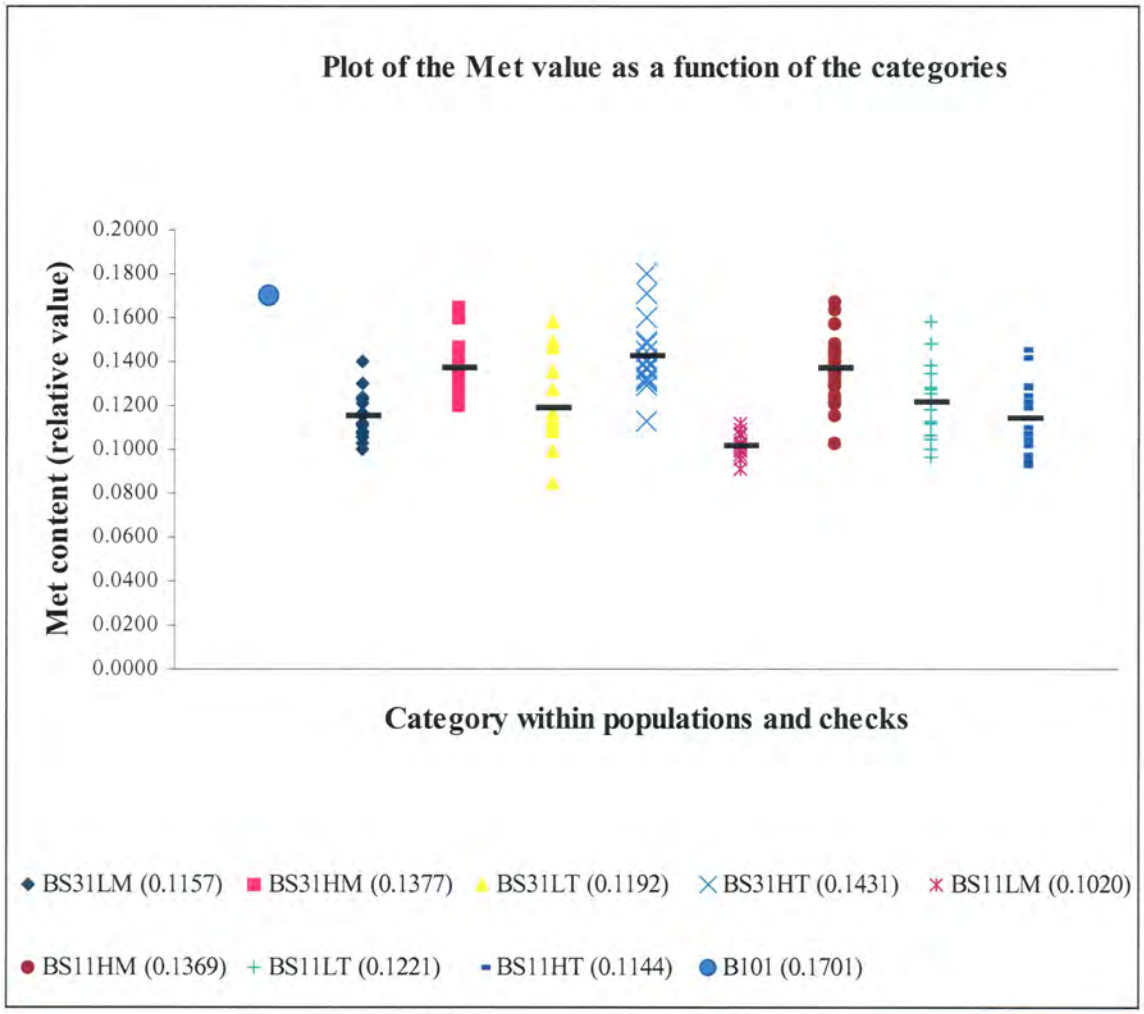

Figure 7. Distribution of the selections from the Intra-mate experiment for each category of BS11 and BS31 populations for their Met content. The check used for its Met content, B101, is also included. The average for each category is represented with a cross bar on the graph and the value indicated in the legend. The lowest selections of the Low Met categories were chosen for the feeding trial, likewise for the selections in the High Met categories.

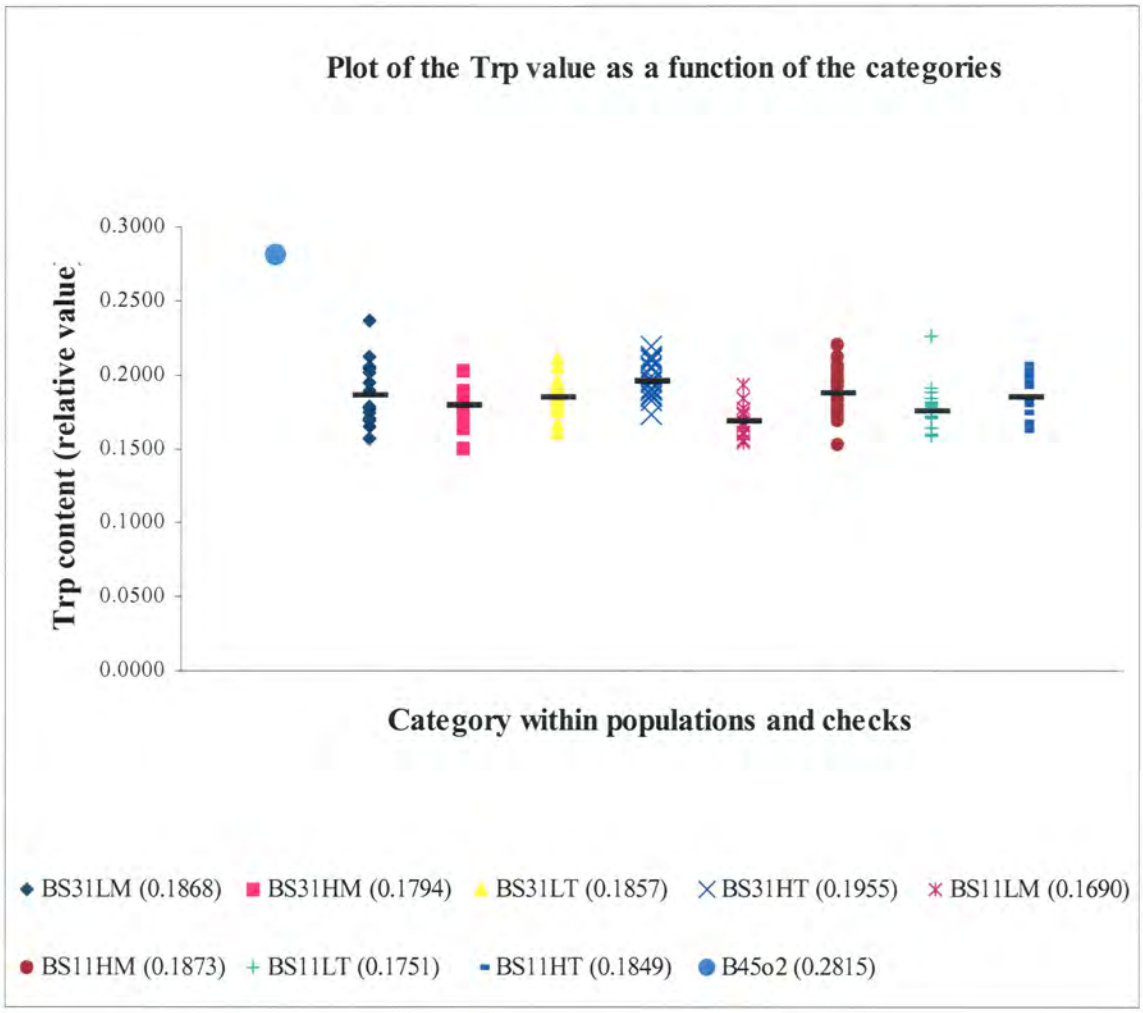

Figure 8. Distribution of the selections from the Intra-mate experiment for each category of BS11 and BS31 populations for their Trp content. The check used for its Trp content, B45o2, is also included. The average for each category is represented with a cross bar on the graph and the value indicated in the legend. The lowest selections of the Low Trp categories were chosen for the feeding trial, likewise for the selections in the High Trp categories.

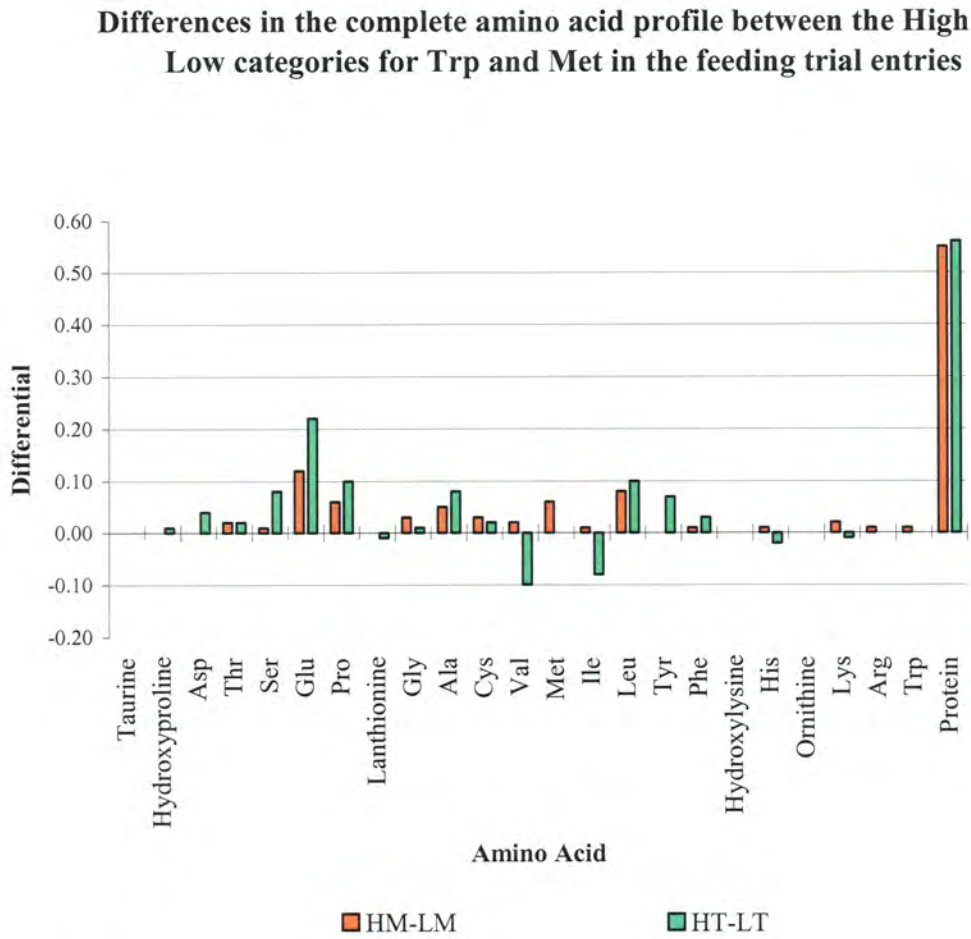

Figure 9. Comparison of the complete amino acid profile of the High and the Low Trp and Met categories in the feeding trial entries. In orange are the differentials for the Met selections and in green, the differentials for the Trp selections.

Figure 10. Body weight of female rats represented by the mean of the individuals in the trial for each diet at 4-day intervals for a period of 25 days. When rats attained the desired body weight, 170 ± 6 grams, they were pulled out of the trial and the remaining rats made up the mean value for the following weigh dates. Throughout the trial, the body weight of rats fed on the High Met (HM) diet was not statistically different than that of rats fed on the Low Met (LM) diet.

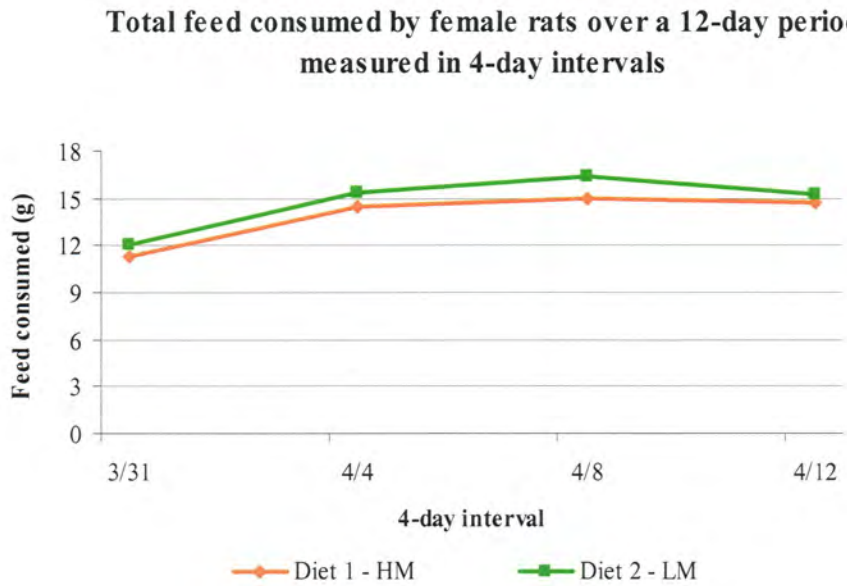

Figure 11. Daily consumption of dietary ration of female rats represented by the mean of the individuals in the trial for each diet at 4-day intervals for a period of 12 days. When rats attained the desired body weight, 170 ± 6 grams, they were pulled out of the trial and the remaining rats made up the mean value for the following weigh dates. Overall, the rats fed on the Low Met (LM) diet consumed more feed than the rats fed on the High Met (HM) diet, with a statistically significant difference at α=0.05.

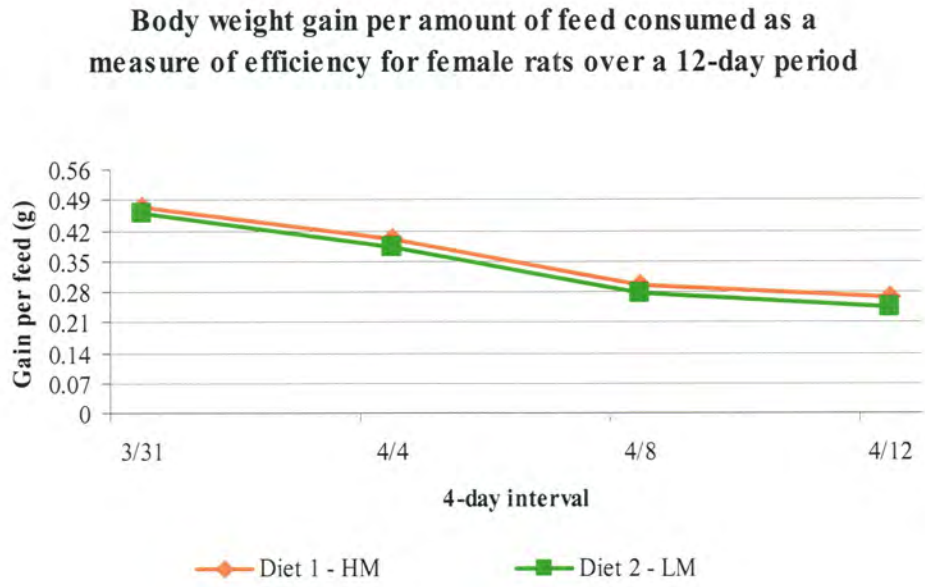

Figure 12. Body weight gain per amount of net feed consumed, or feed efficiency, of female rats represented by the mean of the individuals in the trial for each diet at 4-day intervals for a period of 12 days. When rats attained the desired body weight, 170 ± 6 grams, they were pulled out of the trial and the remaining rats made up the mean value for the following weigh dates. There was no statistical difference in feed efficiency observed in female rats.

Table 1. Mean Met and Trp values for each category of populations derived from BS11 and BS31 for the Inter-mate and Intra-mate experiments (A). Statistical analysis for mean comparisons of the categories for each trait in each experiment (B). All statistically significant differences show that the mean value of the High category is higher than the mean value of the Low category. A notable exception is the contrast HT vs. LT for the Intra-mate experiment, in which the mean value of the Low Trp category is significantly higher than the mean value of the High Trp category.

A. Mean Met and Trp values

			Population[§]	
Experiment	**Trait**	**Category**	**BS11**	**BS31**
Inter-mate	Met	HM	0.1643	0.1757
		LM	0.1533	0.1736
	Trp	HT	0.3030	0.2985
		LT	0.2849	0.2916
Intra-mate	Met	HM	0.1790	0.2039
		LM	0.1678	0.1929
	Trp	HT	0.1404	0.1726
		LT	0.1423	0.1796

§ Relative values for the trait represented by the optical density measurement corrected for the mass of the sample.

B. Statistical analysis for mean comparisons

		Population	
Experiment	**Contrast**	**BS11**	**BS31**
Inter-mate	HM vs. LM	**	n.s.
	HT vs. LT	**	n.s.
Intra-mate	HM vs. LM	**	**
	HT vs. LT	n.s.	**

**, significant at α=0.05
n.s., not significant

Table 2. Complete amino acid profile obtained from the University of Missouri-Columbia Experiment Station Chemical Laboratories for the feeding trial entries. Highlighted are the sulfur-containing amino acids, Cysteine and Methionine, and Tryptophan. The ratio of the sulfur-containing amino acids in HM to that in LM is 0.51 to 0.42, a 17.6% difference.

Type*	HM	LM	HT	LT
Taurine	0.00	0.00	0.00	0.00
Hydroxyproline	0.02	0.02	0.03	0.02
Aspartic Acid	0.65	0.65	0.68	0.64
Threonine	0.34	0.32	0.35	0.33
Serine	0.40	0.39	0.47	0.39
Glutamic Acid	2.15	2.03	2.18	1.96
Proline	0.97	0.91	1.01	0.91
Lanthionine	0.01	0.01	0.00	0.01
Glycine	0.38	0.35	0.37	0.36
Alanine	0.84	0.79	0.85	0.77
Cysteine	**0.25**	**0.22**	0.26	0.24
Valine	0.53	0.51	0.39	0.49
Methionine	**0.26**	**0.20**	0.23	0.23
Isoleucine	0.38	0.37	0.27	0.35
Leucine	1.43	1.35	1.39	1.29
Tyrosine	0.30	0.30	0.34	0.27
Phenylalanine	0.54	0.53	0.52	0.49
HydroxyLysine	0.00	0.00	0.00	0.00
Histidine	0.30	0.29	0.27	0.29
Ornithine	0.01	0.01	0.01	0.01
Lysine	0.29	0.27	0.28	0.29
Arginine	0.45	0.44	0.44	0.44
Tryptophan	0.07	0.06	**0.07**	**0.07**
	10.57	10.02	10.41	9.85

* Balanced bulks of seed represented each of the four types: HM, HT, LM, LT

Table 3. Near-Infrared (NIR) spectroscopy analysis for the feeding trial entries as represented by three readings for each entry.

Type	Protein*	Oil*	Starch*
HM	13.6	5.2	67.3
	14.9	5.1	65.8
	13.3	5.1	67.5
LM	12.4	4.8	68.6
	12.4	5.1	70.0
	13.0	5.2	68.3
HT	12.4	3.9	68.7
	13.1	5.2	66.9
	14.2	4.1	67.8
LT	12.9	4.4	69.2
	11.8	4.9	71.1
	11.8	5.0	67.6

* Dry Matter Basis and corrected for bias

Table 4. Mean contrasts used to characterize the feeding trial entries for the protein, starch, and oil content among the High selections and the Low selections.

Contrast	Mean Square	F Value
HM protein vs. LM protein	2.67	3.87 *
HM starch vs. LM starch	6.62	9.59 ***
HM oil vs. LM oil	0.02	0.02 n.s.
HT protein vs. LT protein	1.71	2.47 n.s.
HT starch vs. LT starch	3.38	4.89 **
HT oil vs. LT oil	0.20	0.29 n.s.

*, **, ***, significant at α=0.10, α=0.05 and α=0.005, respectively
n.s., not significant

Table 5. Diet composition for the rat feeding trial with the corn meal from the selections made from the improved BS11 population. Three batches of three kilograms for each diet were prepared from the same recipe to feed the rats for 25 days.

	Experimental diets	
Ingredient (g)	Diet 1	Diet 2
Corn - **High Met**	2400.0	---
Corn - **Low Met**	---	2400.0
Soy concentrate	300.0	300.0
Lysine	1.5	1.5
Threonine	0.6	0.6
Tryptophan	0.7	0.7
Selenium Mix	1.5	1.5
Corn Oil	90.0	90.0
Starch	104.1	104.1
Calcium	69.0	69.0
Limestone	10.2	10.2
Salt	6.0	6.0
Choline Chloride	9.0	9.0
ISU Mineral Mix	1.5	1.5
Base Vitamin Mix	6.0	6.0

Table 6. Growth performance of female rats fed corn meal improved for its levels of Methionine and supplemented with the necessary amounts of the other essential amino acids, vitamins, and minerals. The average daily gain was calculated from the body weight of rats at four-day intervals and the rats were weighed at the same time in the morning and in the same conditions. The daily net feed consumption accounts for the feed ingested corrected for any waste feed. The efficiency represents the body weight gain per amount of net feed consumed over four-day periods.

Diet	**Avg daily gain** (g)	**Net feed consumed** (g day^{-1})	**Efficiency** (gain g $feed^{-1}$)
HM	4.981 n.s.	13.533 **	0.3589 n.s.
LM	4.965	14.175	0.3398

**, significant at α=0.05
n.s., not significant

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Chapter 3: VARIABILITY AND GENETIC EFFECTS FOR TRP & MET IN COMMERCIAL MAIZE GERMPLASM

A manuscript to be submitted for publication in the journal *Crop Science* or *Maydica*

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1. Abstract

The constituents of maize protein are imbalanced due to limitations in the essential amino acids, Lysine, Tryptophan and Methionine. As maize is a major food and feed crop in many parts of the world, special attention should focus on the improvement of the levels of these limiting amino acids. Before improved maize varieties reach a commercial status, preliminary studies pertaining to the variability of amino acid levels in commercial germplasm and to the genetic effects associated to those amino acids are necessary. This study was conducted in collaboration with a private maize breeding company, Pau Seeds, Inc, a subsidiary company of Bayer Crop Science. The objectives of this study are (1) to determine the variability in Methionine (Met) and Tryptophan (Trp) levels present in commercial maize inbred lines, (2) to characterize the genetic groups of a private maize breeding germplasm for their Met and Trp content, (3) to determine and estimate the genetic effects controlling Trp and Met content, such as general combining ability, specific combining ability, and reciprocal effects, and (4) to establish the potential for breeding for Trp and Met levels. In the first part of the study, seventy-six commercial inbred lines were analyzed for Trp and Met levels. The inbred lines were categorized by their corresponding genetic groups. Significant differences were found within and among the genetic groups,

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hence elucidating the variability for Trp and Met levels in commercial inbred lines. In the second part of the study, two six-parent-diallels for Trp and Met were completed and all of the F_1 crosses and the parents were analyzed for their Trp and Met content. The analyses of variance showed a relatively high general combining ability (GCA) effect for both diallels. The estimates for the GCA effect indicated which inbred lines contributed most to the F1 hybrid in terms of Trp or Met levels. Strong reciprocal and maternal effects were also identified in the analysis. These studies suggest that Met responds better to selection than Trp. Also, maize breeders will be able to exploit the genetic effects in their crossing design to maximize the levels of Trp or Met.

2. Introduction

Maize is a major food and feed crop in many parts of the world. For this reason, improving the nutritional quality of maize is an important objective. Maize is nutritionally limited by deficiencies in Lysine, Methionine, and Tryptophan. Another objective is to establish breeding methods for the selection of nutritionally improved commercial germplasm. In private seed research companies, the demands of high throughput and economic returns are crucial. For those reasons, the preliminary steps are to survey the germplasm utilized in maize breeding and to characterize the different genetic groups that represent the germplasm for their amino acid content.

A number of studies have been conducted with the objective of characterizing grain amino acid levels in maize varietal trials. As early as the 1940's, Doty *et al* (1946) wanted to determine whether or not grain from different single crosses among inbred lines of maize varied in its quality protein content. They concluded that the amount of the amino acids

present in maize kernel protein was related to the genetic constitution of the hybrids. Aguirre *et al* (1953) not only studied the Lysine content of maize but also its Methionine content. They reported genetic variation in the Methionine content of maize, which confirms the findings by Doty *et al* (1946). Tello *et al* (1965) investigated the racial and varietal trends affecting the nutritive value of maize. By sampling a collection of varieties from Mexico, Central America, and the Caribbean, Tello *et al* concluded that Lysine content is a racial characteristic. Zuber *et al* (1975) reported very little differences in mean Lysine content among dent, floury, popcorn, and flint types of maize. None of the accessions representing these four groups had a Lysine content greater than an opaque-2 variety. Goertz *et al* (1978) studied 158 entries from an Andean maize collection and found that while there was an association between protein concentration and grain type, there was no corresponding improvement of percentage of Lysine in the dry matter of grain.

In addition to the racial classification and to the different types of maize, breeders utilize a more specific classification of the germplasm for breeding purposes. Maize germplasm in private breeding companies are commonly classified by their derivation, which may be a population, an inbred line, or a cross (MBS Genetics, 2002). The importance of this classification consists of utilizing the pre-established heterotic patterns from which the progenitor of the populations, inbred lines, or the crosses originated and of developing new heterotic patterns. Maize breeders that devote their research to the development of inbred lines for the production of hybrid seed use the concept of heterotic groups to maximize heterosis in the hybrids (Hallauer, 1997). In the U.S. Corn Belt for example, the Lancaster Sure Crop and the Reid Yellow Dent germplasm represent the two main heterotic groups. Inbred lines obtained from those two heterotic groups tend to complement one another to

maximize hybrid performance. The single-cross hybrid B73 x Mo17 illustrates this pattern. B73 was derived from the Iowa Stiff Stalk Synthetic, a population representative of the Reid Yellow Dent. Mo17 was derived from the cross (187-2 x C103); in which 187-2 is an improved strain of Reid Yellow Dent (Krug) and C103 is a derivative of Lancaster Sure Crop (Hallauer et al., 1988). A common heterotic pattern is Europe includes U.S. dent inbred lines crossed with European flint inbred lines. In Mexico and tropical regions, common heterotic groups include Tuxpeño, ETO composite, and Suwan I (Hallauer et al., 1988).

There exist a number of breeding methods to meet the needs of a breeder, one of which is the diallel mating design. As stated by Dr. Hallauer, "a diallel cross is a mating scheme for developing progenies to determine the genetic action for a group of material" (personal communication). In a diallel mating design, each parent is crossed with every other parent. There are two possible models to analyze a diallel scheme. In model I, or a fixed model, analysis pertains to the use of a selected set of parents or cultivars. In model II, or a random model, analysis is applicable to parents deriving from a random sample of a reference population. For the Model I analysis, information can be obtained on the average performance of the parents in crosses (GCA), specific cross performance (SCA), and the genetic effects of parents and crosses. The concepts of GCA and SCA are useful to plant breeders in characterizing inbred lines in crosses (Hallauer and Miranda, 1986). GCA is defined as the average performance of a line in hybrid combinations and pertains solely to the inbred lines included in the diallel. SCA is defined as the expected performance of a specific hybrid combination when compared to the average performance of the parent inbred lines (Sprague and Tatum, 1942). A complete diallel, where all possible crosses and parents are included, with a Model I analysis was used in the present study and emphasis was

directed toward the genetic effects of parents and crosses. From a complete diallel mating design, one may investigate the following questions pertaining to the genetic effects of traits in particular: additive effect, dominance effect, and reciprocal effect. Griffing (1956) presents four methods for the analysis of variance, each method being specific for the inclusion or not of the parents and the reciprocal crosses in the analysis. His methods examine the concept of combining ability, hence elucidating the GCA and SCA effects.

The objectives of this study are (1) to determine the variability in Met and Trp levels present in commercial maize inbred lines, (2) to characterize the genetic groups of a private maize breeding germplasm for their Met and Trp content, (3) to determine and estimate the possible genetic effects of Trp and Met, such as general combining ability, specific combining ability, and reciprocal effects, and (4) to establish the potential for breeding for Trp and Met levels using currently available commercial germplasm.

3. Materials and methods

3.1. Plant material

The first study consisted of seventy-six different maize inbreds. The inbred lines were categorized according to their genetic group as follows: B14/B73, B14/B73/Iodent, B73, OH43/W153R, OH43/Iodent, Mo17, European Flint, Plata, and Unrelated. Table 1 contains a description of the different genetic groups in terms of agronomic traits. Seeds for each entry were collected from various sources and from different years.

The second study used a subset of the inbred lines used in the first study. Seeds from the selected inbred lines were planted in the summer of 2002. Two complete diallels, hereafter referred to as Trp Diallel and Met Diallel, were designed to include six parents each. The

parental lines from the Trp Diallel were each from a different genetic group and represented the highest or the lowest values of Trp within each genetic group. The choice of whether a high or low inbred line was chosen from a specific group was made arbitrarily. Parents for the Met Diallel were chosen similarly; only they were selected on the basis of their Met content. Two inbred lines, PSI101 and PSI301, were common to both diallels as they had high levels of both Trp and Met. Table 3 provides a description of the parent inbred lines that were included in the two six-parent diallels and their corresponding genetic groups. The inbred lines are proprietary and have been coded for confidentiality purposes. Each parental line was crossed to each other line in the diallels, and reciprocal crosses were made in each case. In addition, each parental line was self-pollinated. Both Trp and Met levels of the resulting kernels were determined, giving two measurements for each ear produced in the diallels (e.g. Trp Diallel - Trp, Trp Diallel - Met, Met Diallel - Met, and Met Diallel - Trp).

3.2. Field procedures

Pau Seeds' breeding nursery is located in Boone, Iowa. The inbred lines used as the parents in the two complete diallels were mostly found in the Parental Line Observation (PLO) plot in the summer of 2002. As the decision to complete a diallel mating design was conceived after planting, the parent inbred lines were not planted in a defined way. Generally, the parent inbred lines were planted in 30-kernel rows and were found scattered within the PLO plot. One plant was used for self-pollination and another plant was used as a male pollen donor or as a female. At harvest, the ears were individually bagged.

3.3. Preparation of samples for analysis of Met and Trp levels

Each ear of maize was shelled and packaged individually. From each ear, five random selected whole kernels were ground using a Wiley Mill with a 40-mesh screen. Each ground sample was stored in an Eppendorf tube. With the flap of the tube open, the samples were then dried for four hours at 65°F, after which the tubes were stored in ambient conditions. Samples were analyzed in 96-well plates using a Randomized Complete Block Design including two checks (B101 and B45o2), and six standards. The B101 inbred was chosen as a check because it has high levels of Methionine (Hallauer and Wright, 1995). The inbred B45o2 was used as a check for high Tryptophan. Commercially-prepared, pure amino acids were used as standard in concentrations of 5, 20, 35, 60, 75, 100 μM for Methionine and 0, 100, 240, 300, 480, 600 μM for Tryptophan. The analysis was replicated on three plates with a different randomization of the entries on each plate (i.e. three blocks). The checks were replicated twice within a plate and the standards three times within a plate. Ten milligrams of the ground sample and check were weighed into the well of a V-bottom, 96-well microtiter plate.

3.4. Protein hydrolysis

Each sample in the plate was subjected to an enzymatic hydrolysis using Pepsin. To each well, 200 μL of 0.1 mg/mL Pepsin solution in a KCl-HCl pH2 buffer was added. The plate was then sealed, covered with a lid, and placed in a 37 °C shaking incubator for approximately 15 hours. After the incubation period, the plate was centrifuged at 3000 rpm for 20 minutes, after which the supernatant was removed for further analysis.

3.5. Assay for Trp

The method for the determination of Tryptophan in maize kernels is a modified version of the one originally described by Sastry and Tummuru (Sastry and Tummuru, 1985). Twenty microliters of hydrolysate or standard was transferred directly into the wells of a flat-bottom, 96-well assay plate. The plates were sealed between operations to prevent evaporation. For each plate, the assay solution consisted of 9.5 mL of concentrated HCl, 250 μL 2.5% Thioglycolic Acid (TGA), and 250 μL 10% sucrose. This solution was prepared and warmed to 42°C for 23 minutes to allow the solution to turn yellow. Under a vented hood, 80 μL of this assay solution was added to the hydrolysate in the assay plate. The plate was then shaken on a plate shaker for three minutes, after which the optical density at 510 nm was determined immediately with a microplate reader.

3.6. Assay for Met

The microbiological method for the determination of Methionine in maize kernels is similar to that described by Wright and Orman (Wright and Orman, 1995). An auxotrophic strain of *Escherichia coli*, P4x (Jacob and Wollman, 1961), was used in this assay. The inoculum was prepared in M9 media supplemented with 10 μL of 1 mg/mL Methionine solution per 5 mL M9 media solution (Maniatis, 1982) and grown to late log phase. Ten μL of hydrolysate or a standard was transferred directly into a flat-bottom, 96-well assay plate. The plates were sealed between operations to prevent evaporation. To each well, 100 μL of M9 media and 2 μL of the inoculum were added. The plate was then sealed, covered with a lid, and placed in a 37 °C shaking incubator for seven hours. After the incubation period, the

plates were shaken on a plate shaker for 3 minutes and the 595 nm light scattered by the sample was determined using a microplate reader.

3.7. Statistical analysis

For the first study, each genotype was considered a treatment. Comparisons among treatment means were determined using an F-test implemented by the GLM procedure of SAS (SAS-Institute, 2000). A Duncan's test was performed to make comparisons among the different genetic groups at the level of significance α=0.05. For the second study, each ear produced in the diallel was considered a treatment, and the mean value of the three measurements for each amino acid was considered an independent variable in the analysis of variance. General combining ability (GCA), specific combining ability (SCA), and the reciprocal effects were calculated for each six-parent diallel using Griffing's Experimental Method 3, Model I, which includes both reciprocal and non-reciprocal F_1 crosses but not the parents (Griffing, 1956). For this model, the restrictions imposed on the combining ability effects are (1) the sum of the estimates of the GCA effects for all parents in the diallel should equal to zero and (2) the sum of the estimates of the SCA effects for all crosses should equal to zero. There is no restriction on the estimates of the reciprocal effects. To estimate the variances of the genetic effects, Griffing (1956) proposes the following formulas to calculate the GCA, the SCA and the reciprocal effects, respectively, where i represent the i^{th} parent, j the j^{th} parent, p the number of parents included in the diallel and σ^2 the error mean square:

$$\text{var}(\hat{g}_i) = \frac{p-1}{2p(p-2)}\sigma^2$$

$$\text{var}(\hat{s}_{ij}) = \frac{p-3}{2(p-1)}\sigma^2 \quad (i \neq j)$$

$$\text{var}(\check{r}_{ij}) = \frac{1}{2}\sigma^2 \quad (i \neq j).$$

In the analysis of variance, the source of variance representing the Entries for each diallel was partitioned to include the parents, the parents versus crosses, and the crosses. The genetic effects GCA, SCA and Reciprocal effects and the Experimental error also were included in the model for the analysis of variance.

4. Results

4.1. Evaluation of inbred lines within genetic groups

In order to characterize the variability for Trp and Met levels in commercial inbred lines, seventy-six inbred lines were analyzed to represent the nine genetic groups that are used to classify commercial germplasm (Figures 1 and 2). At least four lines from each group were examined. The values for each genetic group are summarized in Table 2. As the interest of the study is to rank and compare values, only the relative Met and Trp concentrations are reported. These values represent the optical density (OD) value corrected for the mass of the sample analyzed. The genetic group B14/B73 had the highest mean values for Trp and Met. The genetic group PLATA had the lowest mean value for Trp and the second lowest mean value for Met. The inbred lines B101 and B45o2 were included in the analysis to serve as checks for high Met and Trp, respectively. A few entries were equal to or higher than the B101 check for their Met content. On the contrary, the B45o2 check had no close

competitors from either genetic group. These data illustrate the variability among different genetic groups and the variability within genetic groups for Trp and Met levels.

4.2. Complete diallels

In order to characterize the genetic effects associated with the Trp and Met levels in maize, we analyzed the seed of the parents and of the F_1 crosses generated in the two six-parent diallels. The mean Trp and Met content for all of the entries in the diallels are presented in Tables 4 and 5, respectively. In each instance, the parent mean was higher than the mean of the crosses. In the Trp Diallel – Trp, the parents selected for their HT content had Trp levels higher than the parents selected for their LT content. In the Met Diallel – Met, two of three parents selected for their HM content had Met levels higher than the parents selected for their LM content. The third parent, PSI702, which was selected as a HM parent, actually had the lowest Met content of all the parents in the Met Diallel.

In order to identify factors with significant contributions to the variance of the values, an analysis of variance was conducted using the following sources of variance in the model: Parents, Parents vs. Crosses, Crosses, GCA, SCA, Reciprocal effects, and the Experimental error. The results of this analysis are presented in Table 6. The test for Parent versus Crosses showed significant differences (α=0.05) between the means of the parents and that of the crosses for Trp Diallel – Trp and Met Diallel – Trp only. In all diallels, the mean squares among Parents and among Crosses showed significant differences, confirming the variability for Trp and Met contents in the lines chosen for this study. Genetic effects including the general combining ability (GCA), defined as a measure of the average performance of inbred lines in crosses and is attributable primarily to additive genetic effects, specific combining

ability (SCA), defined as the component of performance that cannot be predicted from additive effects and is due to dominance genetic effects, and the reciprocal effects made significant contributions to the model in all diallels for both traits.

The estimates of the GCA effects, the SCA effects, and the reciprocal effects for Trp Diallel-Trp and Met Diallel-Met are reported in Table 7. Interestingly, more estimates of the genetic effects were found significant in the Met Diallel-Met than for the Trp Diallel-Trp. In the Trp Diallel-Trp, PSI101, a HT parent and a derivative of the B14/B73 genetic group, had the highest and the only significant estimate of the GCA effect. In the Met Diallel-Met, PSI101, a derivative of the B14/B73 genetic group, and PSI702, a derivative of Mo17, both HM parents, had the highest and significant (α=0.01) estimates for the GCA effect.

As the sum of the individual estimates for the SCA effect must equal zero, a positive and statistically significant estimate for the SCA effect represents the best hybrid combination in the diallel for its Met or Trp levels. In the Trp Diallel – Trp, the HTHT combinations (i.e. combinations in which the female HT parent is crossed to a male HT parent) and the LTLT combinations all had negative and sometimes significant estimates for the SCA effect. The most positive and statistically significant estimate for the SCA effect was found in a HTLT combination (PSI301*PSI801). With one exception, all HTLT combinations had positive estimates for the SCA effect. In the Met Diallel – Met, two out of three HMHM combinations had positive and significant estimates for the SCA effect. The highest and significant estimate for the SCA effect was found in a HMLM combination (PSI101*PSI002), though most other HMLM combinations had negative and significant estimates for the SCA effect. The estimates for the SCA effect for the LMLM combinations were either not significant or positively significant.

According to Griffing's analysis, there is no restriction for the estimates for the reciprocal (REC) effects. The estimates for the REC effect may be interpreted as follows: a positive estimate implies that the combination in which a specific parent is used as a female has higher Met or Trp levels than in the same combination in which the parent is used as a male, vice versa for a negative estimate for the REC effect. In the Trp Diallel – Trp, all HTHT combinations had negative, though not significant, estimates for the REC effect. Most HTLT combinations had positive estimates for the REC effect. As for the LTLT combinations, the estimates for the REC effect were positive. The LTLT combination PSI001*PSI401 had the highest and significant estimate for the REC effect, followed by the HTLT combination PSI401*PSI101. In the Met Diallel – Met, two of three HMHM combinations had negative and significant estimates for the REC effect. The highest positive and significant estimates for the REC effect were found in HMLM combinations. All of the LMLM combinations had negative and significant estimates for the REC effect.

Further statistical analyses were conducted to compare the different High-Low combinations, for the Trp Diallel and the Met Diallel. Each cross in the diallel was coded by the traits for which each parent was selected (e.g. HMHM for the cross of a HM parent by another HM parent, where the first parent in the cross was used as the female). Table 8 summarizes the mean values for the diallel entries grouped by the relative amino acid content (High or Low) of the parental lines. Table 9 summarizes the mean squares for the contrasts using these groupings.

For both diallels, Trp Diallel-Trp and Met Diallel-Met, the analyses of variance showed highly significant differences among the four types (e.g. for Met Diallel-Met: HMHM, HMLM, LMHM, LMLM) and the parents. For Trp Diallel-Trp, the overall mean of the

parents was significantly higher than the diallel crosses in which the female parent was selected for its LT content (LTHT and LTHT). When the female parent was selected for its HT content, the mean of the diallel crosses was not significantly different from the overall mean of the parents. This illustrates the maternal effect in which there is a substantial difference in combinations in which a High parent or a Low parent is used as the female. Likewise, for Met Diallel-Met, the overall mean of the parents was significantly higher than the mean of the diallel crosses in which the female parent was selected for its LM content (LMHM and LMLM). When the female parent was selected for its HM content, the mean of the diallel crosses was significantly higher than the mean of the parents. For both diallels, the contrasts between the High combinations (e.g. HMHM) and the Low combinations (e.g. LMLM) were significantly different, with the amino acid content of the High combinations higher than that of the Low combinations.

Also common to both diallels is the highly significant contrast for the reciprocal type of crosses, HTLT vs. LTHT and HMLM vs. LMHM. The cross in which the High parent was used as a female is consistently and significantly higher than the cross in which the Low parent was used as a female. For Trp Diallel – Trp, the mean Trp content for the HTHT type of crosses was significantly lower than the mean Trp content for the HTLT crosses and was not significantly different from the LTHT crosses. For Met Diallel – Met, the mean Met content for the HMHM type of crosses was significantly higher than the mean Met content of the LMHM crosses and was not significantly different from the HMLM crosses. Finally, the mean Trp content of the LTHT crosses was significantly higher than the mean Trp content in the LTLT crosses. The mean Met content of the LMHM crosses was not significantly different from the mean Met content in the LMLM crosses.

5. Discussion

5.1. Variability for Trp and Met levels in commercial germplasm

A total of seventy-six commercial inbred lines representing nine genetic groups were analyzed to determine the extent of variability in Trp and Met levels. Variability was found within and among the genetic groups. The B14/B73 genetic group had both the highest mean Met and Trp values. The Plata genetic group generally had low levels of both Met and Trp. One inbred from the B14/B73/Iodent group and one from the B73 (PSI301) group had substantially higher Met levels than the check B101. No inbred had a higher Trp level than the check B45o2. One of the tenets in plant breeding is the presence of genetic variability in order to pursue an effective selection. As the genetic groups represent germplasm currently in use in commercial breeding programs, these data indicate that breeding for Trp and Met levels with commercial germplasm is feasible.

5.2. Determination of genetic effects for Trp and Met levels from diallel crosses

The significant differences among the parents and among the crosses suggest and confirm the findings that there is great variability in maize germplasm for Met and Trp. The analyses of variance of the diallels showed that the GCA makes the largest contribution to the model, with a more pronounced contribution in the Met Diallel – Met than in the Trp Diallel – Trp. As Sprague and Tatum (1942) demonstrated and as reviewed by Hallauer and Miranda (1986), the GCA is indicative of genes having largely additive effects. Additive effects reflect the average effects of genes. This notion is important to plant breeders when selecting breeding methods, as some selection methods will emphasize GCA more than others. The success of recurrent selection is to select individuals with favorable alleles, thus

high estimates of GCA effects, and incorporate them into the next cycle of selection. From the presentment of the estimates of the GCA effect, we observed that in the Trp Diallel – Trp and the Met Diallel – Met, the highest, significant estimates for the GCA effect were found in a High parent. Whether a maize breeder wants to establish a recurrent selection program for high levels of Trp or Met or develop hybrids with high levels of Trp or Met, an inbred line with a high estimate for the GCA effect should be used to realize the potential of a successful hybrid combination.

In all diallels with the exception of Met Diallel – Met, the SCA and the reciprocal effects contribute about equally to the model, but to a lower extent than the GCA. The SCA is indicative of genes having largely dominance effects. Dominance effects are due to allelic interactions. To the plant breeder, a high estimate of the SCA effect for two specific parents indicates a good hybrid combination. From the presentment of the estimates for the SCA effect, we observed that the hybrid combination with the highest levels of Trp or Met involved a High parent and a Low parent. However, the other estimates for the SCA effect for the High*Low combinations were either not significant (Trp Diallel – Trp) or negatively significant (Met Diallel – Met). As those estimates pertain to those crosses in the diallels only, further investigation is necessary to confirm these SCA effects. As for the reciprocal (REC) effects, the estimates for the REC effect that are of interest are those that pertain to the High*Low combinations. The interpretation of those estimates will determine the way in which a hybrid combination has the highest levels of Met or Trp. We observed that most High*Low combinations in the diallels had positive and sometimes significant estimates for the REC effect. This shows that High*Low combinations in which the High parent is used as

the female had higher levels of Met or Trp. From these analyses, we then can infer a reciprocal effect in the direction of the female parent for both Met and Trp levels.

Heterosis, or hybrid vigor, is a very important concept for plant breeders, especially in breeding cross-pollinated crops. In the analyses of variance, the test of Parents versus Crosses is an indication of heterosis. Interestingly, the Mean Squares for this test were found significant (α=0.05) in Trp Diallel – Trp and Met Diallel – Trp, whereas the same test was non-significant for the Met Diallel – Met and Trp Diallel – Met. This indicates that there is heterosis for Trp levels and none for Met levels. These results may need further investigation, as heterosis not only benefits the breeder but also the end-user.

The reciprocal effects need to be considered carefully also as they will dictate the way in which a cross needs to be made in order to optimize the trait of interest. To confirm the findings from the estimates of the reciprocal effect in which we concluded a reciprocal effect in the direction of the female parent for both Met and Trp levels, mean comparisons were completed for the combinations in which each inbred was classified as either a High or Low parent for the amino acid examined in the diallel. This analysis confers additional information about the possible genetic effects. The contrasts HTLT vs. LTHT and HMLM vs. LMHM reveal a very strong reciprocal effect, which confirms the previous analysis of variance. This reciprocal effect may also illustrate the maternal effect. Specific to Met Diallel – Met, the mean Met content of the crosses of type HMHM was not significantly different from the mean Met content of the HMLM crosses. However, the mean Met content of the HMHM crosses was significantly higher than the mean Met content of the LMHM crosses. Again, this may confirm the maternal effect with the LM females contributing less to the hybrid seed in Met content than the HM females. In addition, all diallel crosses in

which the LM parent was used as a female had a significantly lower mean Met content than the mean content of the parents. Finally, all diallel crosses in which the HM parent was used as a female had a significantly higher mean Met content than the mean content of the parents. Such observations were not obvious in the Trp Diallel – Trp. The only similar trend associated with Trp is that the crosses involving LT as a female parent had a significantly lower mean Trp content than the mean Trp content of the parents. Although no significant differences were found in the first analysis of variance involving the Parents vs. Crosses for Met, grouping the crosses according to the relative amino acid content of the parents revealed significant heterosis for Met content in F_1 seed.

These results may be useful for maize breeders. The genetic effects associated with Trp levels seemed of lesser amplitude than those associated with Met levels. This may be due to less variability in Trp levels in maize germplasm and, or to the analytical method used to quantify Trp in maize kernels. From the standard errors computed for the estimates of the genetic effects, the precision of the assays was quite different. Overall, the standard errors for the Trp assay were twice as great as for the Met assay.

The genetic effects associated with Met were interesting to decipher. A strong reciprocal effect and an obvious maternal effect will facilitate the breeder in making decisions for the crosses to make, using a High Met inbred line as the female parent. Also, the high overall GCA and the estimates of the GCA effect specific to the parents in the diallel will provide information to the breeder as to which inbred parent to cross to which testers in order to monitor not only the amino acid content, but also the performance of the hybrid in terms of yield. The female parent can contribute high levels of Met and the male parent can contribute other traits of interest such as yield or disease resistance, for example. The

distinct variability in Trp and Met levels among the genetic group will also permit the breeder to recycle inbred lines and maintain genetic variability within a genetic group while enhancing the germplasm for high levels of Trp or Met.

6. Figures and Tables

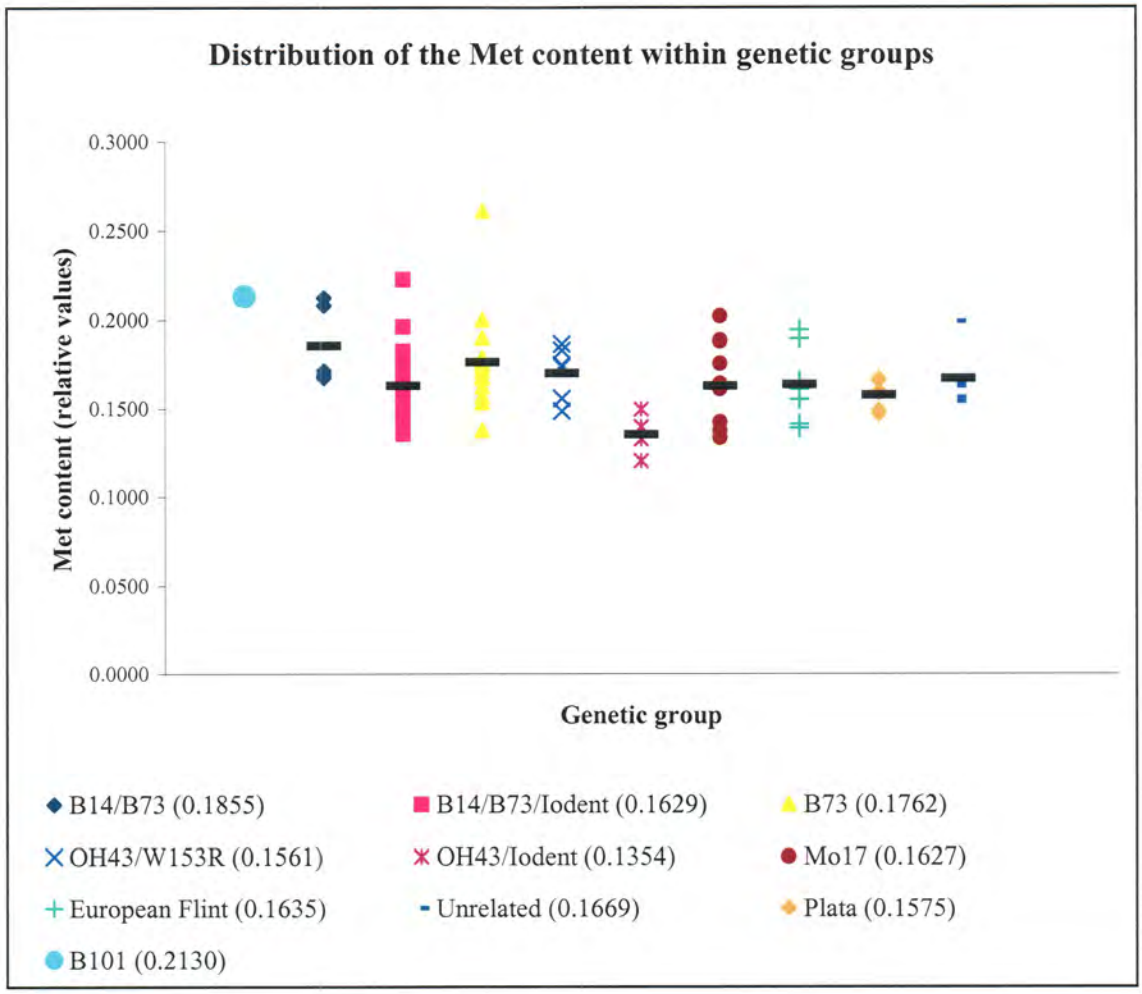

Figure 1. Distribution of the nine genetic groups and the check B101 for their Met content. The mean Met content (relative value) for each genetic group is represented by the cross bar in the graph and indicated in the legend.

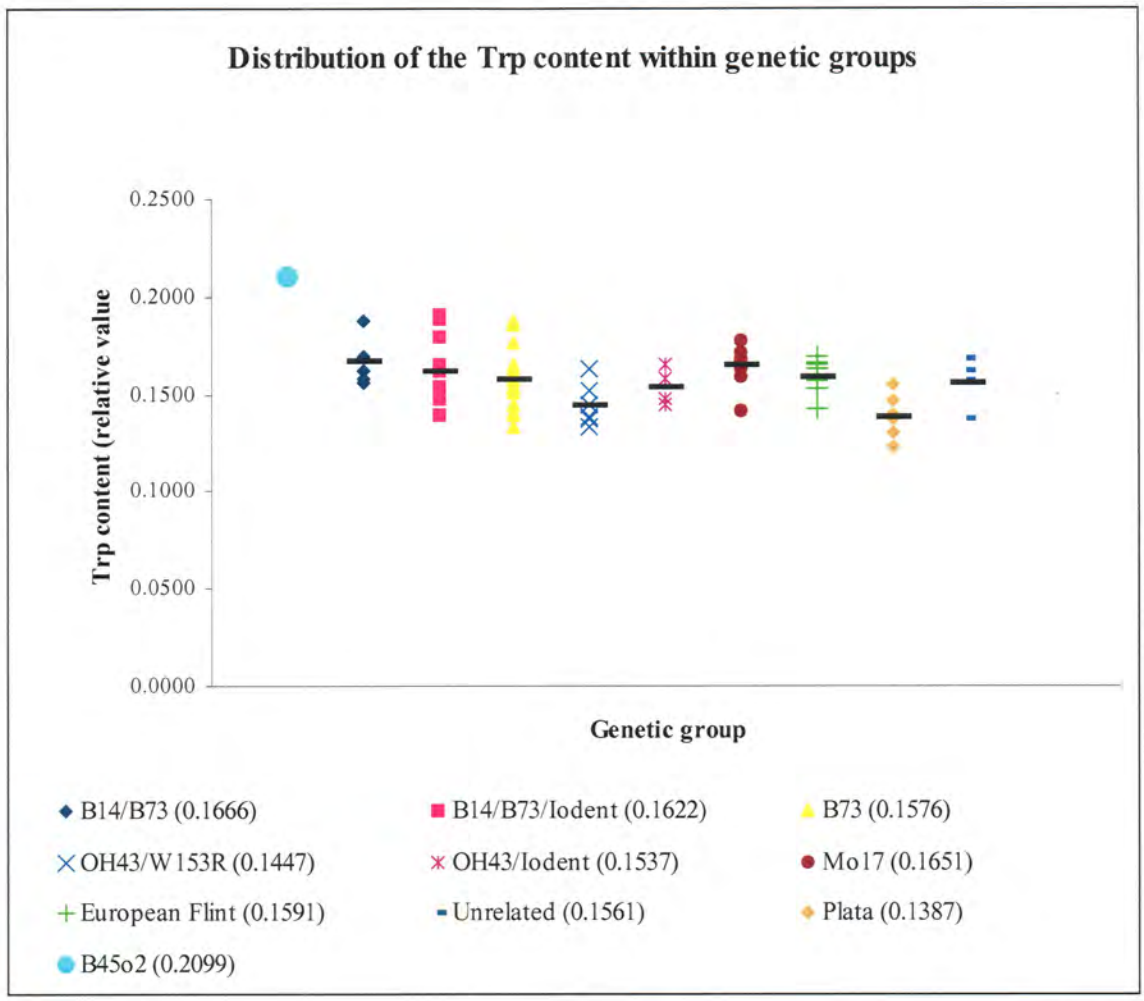

Figure 2. Distribution of the nine genetic groups and the check B45o2 for their Trp content. The mean Trp content (relative value) for each genetic group is represented by the cross bar in the graph and indicated in the legend.

Table 1. Description of the different genetic groups in terms of agronomic traits (Christian Buffard, personal communication).

Genetic Group	Characteristics
B14/B73	Seed parent – Larger ear type – Average kernel rows number - Dented kernel type – Average root strength – Good stalk efficiency- Lower tolerance to leaf diseases – Fast dry down – Average plant integrity late season
B14/B73/Iodent	Similar to previous group – Iodent enhances earlier flowering and faster dry down – Late season plant intactness is also improved.
B73	Seed parent – Large girthy ear type – Dented kernel – Average test weight – Taller pant stature - Very good stalk and root strength – Good stay green but susceptibility to the European Corn Borer – Later flowering, slower dry down.
OH43/W153R	Pollinator – Determined ear type – Softer kernel texture – High stress tolerance (heat, drought) – Shorter plant stature – Average root quality – Good leaf diseases tolerance – Earlier flowering and fast dry down.
OH43/Iodent	Pollinator – Semi-determined ear type – Improved test weight over OH43/W153R – Medium plant size – Good stalk strength – Early flowering and very efficient dry down – Very good plant integrity in late season.
Mo17	Pollinator – Longer, slender ear type with good flex – Harder kernel texture (more flinty, more round in grade out than B73's) and higher test weight – Taller plant height – Good stalk strength and average root efficiency – Late flowering and slower dry down – Good stay green and leaf diseases tolerance – Better tolerance to the European Corn Borer.
European Flint	Pollinator – Small ear, few kernel rows – Vitreous round kernel type (flint), higher test weight – Very early flowering and very early grain set in the fall but slow dry down after black layer – Good cold tolerance at emergence.
Plata	Pollinator used in the U.S. - Late Argentine-based germplasm – Flinty kernel texture – Very high test weight – Late flowering and very slow dry down in U.S. temperate conditions – Very good stalk quality and average root strength – Good tolerance to diseases and ear damaging insects.
Unrelated	Mix of varied origins above.

Table 2. Mean values of the genetic groups and the checks for their Trp and Met values. The Met and Trp values represent the optical density corrected for the mass of the sample analyzed. The number of entries represents the number of inbred lines that were included in each genetic group and that went into the mean values.

		Trp value		Met value	
Genetic Group	**Number of entries**	**Avg**	**Stdev**	**Avg**	**Stdev**
B101 (Met check)	1	.	.	0.2130 a	.
B45o2 (Trp check)	1	0.2099 a	.	.	.
B14/B73	5	0.1665 b	0.0129	0.1855 a	0.0226
Mo17	10	0.1651 b,c	0.0097	0.1627 b,c	0.0249
B14/B73/Iodent	16	0.1622 b,c	0.0163	0.1628 b,c	0.0230
European Flint	8	0.1591 b,c	0.0086	0.1635 b,c	0.0200
B73	16	0.1576 b,c,d	0.0154	0.1763 b	0.0268
Unrelated	5	0.1561 b,c,d	0.0114	0.1669 b,c	0.0183
OH43/Iodent	4	0.1537 b,c,d	0.0096	0.1354 c	0.0122
OH43/W153R	6	0.1446 c,d	0.0108	0.1700 b,c	0.0151
Plata	6	0.1387 d	0.0113	0.1575 b,c	0.0084

a, b, c, d represent different Duncan groupings. Entries in the same grouping are not statistically different (α=0.05).

Table 3. Description of the parent inbred lines included in the diallels with the genetic group in which they belong and the trait for which they were selected, high Trp (HT), low Trp (LT), high Met (HM), and low Met (LM). Two inbred lines, PSI101 and PSI301, were common to the two diallels as they had high levels of both Trp and Met.

Diallel	**Parent inbred line**	**Genetic group**	**Trait**
Trp	PSI101	B14/B73	HT (HM)
	PSI301	B73	HT (HM)
	PSI701	Mo17	HT
	PSI801	OH43/W153R	LT (HM)
	PSI401	B14/B73/Iodent	LT
	PSI001	Unrelated	LT
Met	PSI101	B14/B73	HM (HT)
	PSI702	Mo17	HM (HT)
	PSI301	B73	HM (HT)
	PSI402	B14/B73/Iodent	LM
	PSI601	OH43/Iodent	LM
	PSI002	Unrelated	LM

Table 4. Mean Trp content (relative value) for two complete diallels, thereby referred to as Trp Diallel - Trp and Met Diallel - Trp. Each diallel includes six maize inbred lines of distinctly different genetic groups and their corresponding reciprocal and non-reciprocal F_1 crosses produced in the summer of 2002 in Boone, Iowa.

Trp Diallel -Trp

♀	♂						
	PSI101	PSI301	PSI701	PSI801	PSI401	PSI001	Mean
PSI101	**0.3364**	0.3086	0.3323	0.3106	0.3065	0.3159	0.3148
PSI301	0.3082	**0.3556**	0.3206	0.319	0.3155*	0.313	0.3152
PSI701	0.3150	0.3155*	**0.3525**	0.3191	0.3254	0.2951	0.3078
PSI801	0.3416	0.3423	0.2956	**0.3281***	0.2771	0.2907	0.3142
PSI401	0.3477	0.3242	0.3168	0.3071	**0.2989**	0.2816	0.3155
PSI001	0.3407	0.3272	0.3309	0.296	0.3245	**0.2970**	0.3239
Mean	0.3306	0.3256	0.3239	0.3057	0.3084	0.2993	
						Grand mean	0.3154
						Parent mean	0.3281

* Missing data point represented by the overall mean of either the parents or the crosses.

Met Diallel - Trp

♀	♂						
	PSI101	PSI702	PSI301	PSI601	PSI002	PSI402	Mean
PSI101	**0.3364**	0.333	0.3086	0.2556	0.2947	0.3295	0.3043
PSI702	0.3175	**0.3169**	0.2824	0.2763	0.2832	0.2858	0.2890
PSI301	0.3082	0.2937	**0.3556**	0.3019*	0.2869	0.3019*	0.2917
PSI601	0.3084	0.2816	0.2889	**0.2961**	0.3048	0.3019*	0.2955
PSI002	0.3542	0.3213	0.2751	0.2866	**0.3075**	0.3433	0.3111
PSI402	0.3452	0.2998	0.2927	0.2871	0.3067	**0.2837**	0.3294
Mean	0.3267	0.3059	0.2926	0.2769	0.3002	0.3004	
						Grand mean	0.3020
						Parent mean	0.3160

* Missing data point represented by the overall mean of either the parents or the crosses.

Table 5. Mean Met content (relative value) for two complete diallels, thereby referred to as Trp Diallel - Met and Met Diallel - Met. Each diallel includes six maize inbred lines of distinctly different genetic groups and their corresponding reciprocal and non-reciprocal F_1 crosses produced in the summer of 2002 in Boone, Iowa.

Trp Diallel - Met

♀	♂						
	PSI101	PSI301	PSI701	PSI801	PSI401	PSI001	Mean
PSI101	**0.2097**	0.1959	0.1796	0.2012	0.1883	0.2103	0.1951
PSI301	0.1925	**0.2185**	0.1911	0.2061	0.2005*	0.2334	0.2058
PSI701	0.1834	0.2005*	**0.1811**	0.2006	0.1698	0.2164	0.1925
PSI801	0.1899	0.2397	0.2003	**0.2046***	0.1804	0.2014	0.2024
PSI401	0.2145	0.2051	0.1751	0.2090	**0.1842**	0.2268	0.2061
PSI001	0.1976	0.2064	0.2061	0.2023	0.1904	**0.2293**	0.2006
Mean	0.1956	0.2118	0.1905	0.2038	0.1822	0.2177	
						Grand mean	0.2003
						Parent mean	0.2046

* Missing data point represented by the overall mean of either the parents or the crosses.

Met Diallel - Met

♀	♂						
	PSI101	PSI702	PSI301	PSI601	PSI002	PSI402	Mean
PSI101	**0.2097**	0.2326	0.1959	0.1497	0.1838	0.1642	0.1852
PSI702	0.2003	**0.1368**	0.2130	0.1439	0.1706	0.1634	0.1782
PSI301	0.1925	0.1851	**0.2185**	0.1801*	0.1623	0.1801*	0.1748
PSI601	0.1927	0.1938	0.1545	**0.1932**	0.1756	0.1801*	0.1777
PSI002	0.2342	0.1890	0.1725	0.1483	**0.1741**	0.1783	0.1765
PSI402	0.1919	0.2074	0.1694	0.1485	0.1485	**0.154**	0.1925
Mean	0.2023	0.2016	0.1904	0.1491	0.1762	0.1588	
						Grand mean	0.1803
						Parent mean	0.1811

* Missing data point represented by the overall mean of either the parents or the crosses.

Table 6. Analyses of Variance on the mean Met and Trp content of self and F_1 seed produced by six maize inbred lines in two diallels for the following sources of variation: parents, parents versus crosses, crosses, general combining ability (GCA), specific combining ability (SCA), and the reciprocal effects.

			Trait	
			Met	Trp
Diallel	**Sources of variation**	**DF**	**Mean Squares**	**Mean Squares**
Trp	Parents	5	0.000360 ****	0.000648 ***
	Parents vs. Crosses	1	0.000083 n.s.	0.000793 **
	Crosses	29	0.000252 ****	0.000302 **
	GCA	5	0.000598 ****	0.000309 *
	SCA	9	0.000189 ***	0.000295 *
	Reciprocal Effects	15	0.000175 ***	0.000303 **
	Experimental Error	79	0.000060	0.000152
Met	Parents	5	0.001022 ****	0.000699 ***
	Parents vs. Crosses	1	0.000005 n.s.	0.001000 **
	Crosses	29	0.000550 ****	0.000511 ****
	GCA	5	0.001313 ****	0.001238 ****
	SCA	9	0.000196 ****	0.000352 *
	Reciprocal Effects	15	0.000508 ****	0.000363 **
	Experimental Error	74	0.000044	0.000184

*, **, ***, **** Significant at α=0.10, α=0.05, α=0.01, α=0.001 respectively

Table 7. Estimates for the genetic effects of the Trp Diallel-Trp (A) and the Met Diallel-Met (B). The estimates for the general combining ability (GCA) are included in the diagonal of the table (embolden). The estimates for the specific combining ability (SCA) are included below the diagonal. The estimates for the reciprocal effects (REC) are included above the diagonal. For the Trp Diallel-Trp, the standard errors for the estimates of the GCA, SCA and REC effects are 0.00398, 0.00478, 0.00872, respectively. For the Met Diallel-Met, the standard errors for the estimates of the GCA, SCA and REC effects are 0.00213, 0.00256, 0.00467, respectively. The first three parents represent the parents with high levels of Trp or Met (High parents) and the other three parents represent the parents with low levels of Trp or Met (Low parents).

A. Trp Diallel-Trp

♀	♂					
	PSI101	**PSI301**	**PSI701**	PSI801	PSI401	PSI001
PSI101	**0.00904 ***	-0.00020	-0.00865	0.01550	0.02060 *	0.01240
PSI301	-0.02104 **	**0.00492**	-0.00255	0.01165	0.00435	0.00710
PSI701	-0.00231	-0.00379	**0.00144**	-0.01175	-0.00430	0.01790 *
PSI801	0.00854	0.01722 **	-0.00261	**-0.00696**	0.01500	0.00265
PSI401	0.00613	0.00300	0.00773	-0.01287 **	**-0.00355**	0.02145 *
PSI001	0.00868	0.00460	0.00098	-0.01027 *	-0.00399	**-0.00490**

*, ** Significant at α=0.05 and α=0.01, respectively

B. Met Diallel-Met

♀	♂					
	PSI101	**PSI702**	**PSI301**	PSI601	PSI002	PSI402
PSI101	**0.01712 ** **	-0.01615 **	-0.00170	0.02150 **	0.02520 **	0.01385 **
PSI702	0.00693 **	**0.01232 ** **	-0.01395 **	0.02495 **	0.00905	0.02200 **
PSI301	-0.00358	0.00608 *	**0.00057**	-0.01280 **	0.00510	-0.00535
PSI601	-0.00930 **	-0.00685 **	0.00335	**-0.01670 ** **	-0.01365 **	-0.01580 **
PSI002	0.01648 **	-0.00778 **	-0.00858 **	0.00325	**-0.00468 ***	-0.01490 **
PSI402	-0.01053 **	0.00163	0.00273	0.00955 **	-0.00338	**-0.00863 ** **

*, ** Significant at α=0.05 and α=0.01, respectively

Table 8. Mean and standard deviation values for the diallel crosses and the parents included in the diallels. The type of crosses, i.e. HTHT, corresponds to the trait for which each parent was initially selected, the female written first. The Parents represent all six parents in the diallel.

Diallel	Type	Mean	Std Dev
Trp	HTHT	0.315522	0.027032
	HTLT	0.333266	0.021440
	LTHT	0.309645	0.020289
	LTLT	0.297517	0.023559
	Parents	0.328087	0.034670
Met	HMHM	0.202176	0.023766
	HMLM	0.195392	0.021605
	LMHM	0.165915	0.016530
	LMLM	0.159853	0.016929
	Parents	0.183665	0.029712

Table 9. Contrasts among the different types of crosses in the diallels and between the parents and the types of crosses. Embolden are the highly significant contrasts.

Diallel	Contrast	Mean Squares
Trp	HTHT vs. HTLT	0.003497 **
	HTHT vs. LTLT	0.003335 **
	HTLT vs. LTHT	0.008612 ****
	LTHT vs. HTHT	0.000402 n.s.
	LTHT vs. LTLT	0.002044 *
	HTHT vs. Parents	0.001292 n.s.
	HTLT vs. Parents	0.000265 n.s.
	LTHT vs. Parents	0.003507 **
	LTLT vs. Parents	0.008626 ****
Met	HMHM vs. HMLM	0.000516 n.s.
	HMHM vs. LMLM	0.015673 ****
	HMLM vs. LMHM	0.012073 ****
	LMHM vs. HMHM	0.016874 ****
	LMHM vs. LMLM	0.000379 n.s.
	HMHM vs. Parents	0.003219 ***
	HMLM vs. Parents	0.001369 *
	LMHM vs. Parents	0.003535 ***
	LMLM vs. Parents	0.004518 ***

*, **, ***, **** Significant at α=0.10, α=0.05, α=0.01, α=0.001 respectively
n.s. not significant

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Chapter 4: GENERAL DISCUSSION

1. General discussion

With the extensive use of maize as a feed or as food, its nutritional qualities could be improved to provide a more balanced diet to animals or humans without, or lessening, the use of dietary supplements. In order to improve the amino acid balance of maize, hence improving its nutritional qualities, a maize breeder needs to be knowledgeable of the effective breeding methods, of the genetic variability for the amino acids of interest present in the germplasm to be utilized, and of the genetic effects associated with the specific amino acid. Such an accomplishment necessitates a multi-disciplinary collaboration among the breeder, the biochemist, and the animal scientist. The role of the biochemist is to develop effective analytical methods for the determination of the amino acid content. The animal scientist will contribute to the prospect of producing nutritionally enhanced varieties by testing the nutritional quality with feeding trials.

In the first paper entitled "Nutritional improvement of maize populations by recurrent selection for amino acid levels", recurrent selection was an effective breeding method for selecting for Methionine and Tryptophan levels. In the two populations used in the study, BS11 and BS31, there was enough genetic variability for Methionine levels to initiate a successful divergent selection. The response to selection for differing Tryptophan levels was not as considerable in either population. This response may be due to little genetic variability for Tryptophan levels in the initial population, genetic drift, or the complexity of the trait that makes it difficult to measure. In addition to testing the potential of recurrent selection, the nutritional quality of the improved BS11 population for Methionine was also tested with a rat

feeding trial. While the rats fed on the Low Met diet consumed significantly more than the rats fed on the High Met diet, there was no difference in weight gain or in feed efficiency for the rats fed on either the High Met diet or the Low Met diet. This showed that the rats fed on the Low Met diet consumed more feed to meet the dietary requirements of protein than the rats fed on the High Met diet.

In the second paper entitled "Variability and genetic effects for Met and Trp in commercial maize germplasm", a collection of inbred lines representing the commercial germplasm of a maize breeding company was characterized for its variability in Methionine and Tryptophan levels. The inbred lines were classified according to their genetic groups. Significant differences in Methionine and Tryptophan levels were found among and within the genetic groups. In addition to the substantial variability for Methionine and Tryptophan levels in commercial germplasm, a diallel mating design was completed to determine the genetic effects associated with those two traits. Strong GCA (general combining ability) effects and reciprocal effects were found as a result of this study. Using a female parent with high levels of either Methionine or Tryptophan will result in a superior hybrid combination in terms of those specific amino acids. Again, this reciprocal effect was mostly significant when associated with the Methionine levels. Maize breeders will be able to optimize the desired increase in Methionine levels by planning their crosses with a female parent of high Methionine levels. They will also be able to select inbred lines and cross them according to the variability in Methionine levels present in the genetic groups. The comprehension of such variability and genetic effects will facilitate maize breeders in producing varieties with enhanced Methionine levels.

The findings of this study suggest a strategy that can be used for producing high Methionine hybrids using existing commercial inbred lines. The first step is to form synthetic populations with inbred lines selected from the genetic groups that were characterized with high levels of Methionine. The next step is to initiate a recurrent selection program, selecting the progenies with the highest levels of Methionine and intermating those selections for the next cycle of selection. Lines would then be derived from the improved populations and incorporated into a crossing program to produce top-crosses, using the newly developed high Methionine lines as the female parent and the different testers as the male parent. While pursuing multiple cycles of recurrent selection for high Methionine levels, agronomic traits could be evaluated concurrently in yield trials and the nutritional value of the improved populations could be evaluated in feeding trials. The anticipated outcome of such a breeding program would be varieties with enhanced levels of Methionine with good, if not superior, agronomic traits.

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