GENETIC AND DIETARY INTERACTIONS OF FISHY-EGG TAINT IN BROWN-SHELLED LAYING HENS

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By

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ABSTRACT

Fishy-egg tainting has long been a problem associated with feeding canola meal (CM) to brown-shelled laying hens. It is a classical example of nutrigenetics, as both dietary and genetic factors must be present for a hen to lay fishy-tainted eggs. Trimethylamine (TMA), the compound responsible for the fishy smell, is produced by bacterial fermentation of choline in the lower gut. CM contains large amounts of choline in the form of sinapine. Choline must first be hydrolyzed from sinapine before it can be absorbed or converted to TMA. Normally, the malodourous TMA is metabolized to the odourless trimethylamine *N*-oxide (TMAO) by flavin-containing monooxygenase 3 (FMO3). A mutation in *FMO3 (c.984A>T)* prevents TMA from being oxidized to TMAO, and subsequently TMA accumulates in the developing egg yolks. Our objective was to determine the inheritance pattern of fishy-egg tainting when hens are fed canola meal, reflecting typical industry conditions. In the first of two trials, hens of a commercial brown-shelled strain were genotyped at *FMO3 c.984A>T* and fed graded levels of CM (0, 6, 12, or 18%). These hens were bred to produce a second generation of hens, which were also genotyped and fed graded levels of CM (0, 6, 12, 18, or 24%) or choline chloride (0, 0.055, 0.110, 0.165, or 0.220%). Choline chloride, at levels up to 0.220%, does not lead to the production of fishy tainted-eggs. When fed CM, TT hens laid fishy-tainted eggs. Mean yolk TMA concentration was not significantly different between hens of the AA and AT genotypes, with means from both genotypes remaining below the human detection threshold for all of the dietary treatments. Large day-to-day variations in yolk TMA concentration were seen in hens of all three genotypes. We concluded that fishy-egg tainting is recessive when hens are fed CM at levels reflecting typical commercial practices.

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Dedication

This thesis is dedicated to my parents, who have always encouraged me to follow my dream.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

LIST OF APPENDICIES

LIST OF ABBREVIATIONS

ADG	Average daily gain
ADI	Average daily intake
bp	Base pair
CM	Canola meal
cm	Centimetre
CV	Coefficient of variation
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FMO	Flavin-containing monooxygenase
HCl	Hydrochloric acid
HWE	Hardy-Weinberg equilibrium
KCl	Potassium chloride
kg	Kilogram
KOH	Potassium Hydroxide
LSD	Least significant difference
M	Molar
mg	Milligram
Mbp	Mega base pairs
ml	Millilitre
µl	Microlitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
nm	Nanometre
OZT	(-)-5-vinyl-2-oxazolidinethione
PCR	Polymerase chain reaction

pmol	Picomole
RSM	Rapeseed meal
RPM	Revolutions per minute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TCA	Trichloracetic acid
TE	Tris-EDTA
TMA	Trimethylamine
TMAO	Trimethylamine *N*-oxide

1 INTRODUCTION

Canola meal (CM) is an economical feed ingredient commonly used in laying hen diets. Though it has a lower nutrient density than soybean meal, CM's lower price and good amino acid balance make it a popular protein source in Canadian, European, and Australian poultry production (Hickling 2001). With the demand for canola oil steadily increasing due to the advent of bio-diesel production, it is expected that the price of CM will decline, making it even more attractive to poultry producers (BBI Biofuels Canada 2006).

The drawback of feeding CM to brown-shelled laying hens is that it causes the production of fishy-tainted eggs (Goh *et al.* 1984, Perez-Maldonado and Barram 2004). Consequently, CM is only included at low levels, if at all, in brown-shelled layer diets. Although brown-shelled eggs only make up a small portion of the total Canadian egg production, they form the majority of the market in areas such as Europe and Australia.

Fishy-egg taint is an example of nutrigenetics, an up and coming field of study examining the effect of genotype on the response of an organism to dietary components (Pérusse 2008). It should not be confused with the often-overlapping field of nutrigenomics, the study of the effects of nutrients on gene expression.

The purpose of this study was to determine the mode of inheritance of fishy-egg tainting under typical production conditions. This information will enable commercial breeding companies to produce taint-free hens, allowing producers to fully utilize CM as an economical feed ingredient.

2 LITERATURE REVIEW

2.1 Trimethylamine

Trimethylamine (TMA), $(CH_3)_3N$ (Figure 2.1a), is a tertiary amine that has a characteristic smell of rotting fish (Emmanuel *et al.* 1984, Hernandez *et al.* 2003). It is highly volatile, with a boiling point of 2.87 ˚C (Aston *et al.* 1944). It is the compound responsible for the fishy smell of tainted eggs (Hobson-Frohock *et al.* 1973).

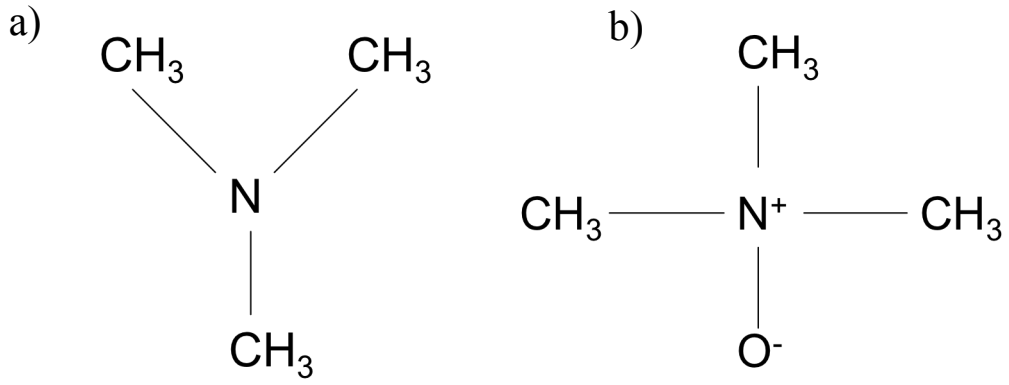

Figure 2.1 Diagrammatic representation of a) trimethylamine and b) trimethylamine *N*-oxide.

Trimethylamine *N*-oxide (TMAO), $(CH_3)_3NO$ (Figure 2.1b), is an odourless metabolite of TMA (Pearson *et al.* 1979). The melting point is 257 ˚C; therefore it is non-volatile (O'Neil *et al.* 2001). The nitrogen-oxygen bond is semipolar in nature, and subsequently the TMAO is salt-like and less basic than TMA (Bickel 1969).

Whereas TMAO is readily filtered from the blood by the kidneys and excreted in the urine, TMA is more difficult to excrete and can subsequently accumulate in circulation and be deposited in developing follicles. It is this accumulation of TMA that leads to fishy egg taint (Pearson *et al.* 1979).

2.1.1 Chickens

TMA can be found in follicular yolks of all sizes in the ovaries of taint-producing hens (March and MacMillan 1979). It has been shown that at least five days are necessary for tainted eggs to be produced after the introduction of a taint-inducing diet (March and MacMillan 1979). This delay was due to the time necessary for TMA to accumulate in the yolk to a level high enough to be detected by smell.

The first noted report of eggs with a fishy taint was by Vondell in 1932. In a later report, Vondell (1948) stated that even though the diet is usually blamed as the source of the taint, "it apparently has no relation to the problem." However, subsequent studies have demonstrated that egg tainting is dependant upon both dietary and genetic factors (Bolton *et al.* 1976, Washburn 1990).

The production of tainted eggs is characteristic to individual hens, and such hens are often found to have fishy-smelling breath (Vondell 1948, Miller *et al.* 1972). The frequency upon which affected hens lay tainted eggs is variable; the eggs from some hens are consistently tainted, while those from other hens are tainted only sporadically (Vondell 1948). Bolton *et al.* (1976) found that approximately one in five eggs produced by a tainting hen is tainted. The frequency of tainted eggs produced by a flock generally decreases as the flock ages (Dänicke *et al.* 2006) and the occurrence has been

reported to be more prevalent during fall and early winter and declines into the spring (Vondell 1948, Miller *et al.* 1972).

2.1.2 Humans

In humans, trimethylaminuria (an accumulation of TMA that is also known as fishy-odour syndrome) is a recessively inherited condition (Dolphin *et al.* 1997a) and is caused by mutations in the *flavin-containing monooxygenase 3* (*FMO3*) gene. There are currently 16 known causative mutations in *FMO3* that result in fishy-odour syndrome in humans, including three nonsense, 12 missense, and one large deletion (Hernandez *et al.* 2003).

Clinical diagnosis of trimethylaminuria is dependant upon urinary levels of TMA (unmetabolized) in comparison to TMAO (metabolized). In unaffected individuals, TMA composes 0 to 9% of total urinary trimethylamines (*i.e.* TMA + TMAO), whereas TMA comprises greater than 40% of the total urinary trimethylamines in individuals with severe trimethylaminuria (Cashman *et al.* 2003).

The mutations in *FMO3* significantly reduce or eliminate the ability of the liver enzyme FMO3 to oxidize TMA into TMAO. Affected individuals secrete TMA in their breath, sweat and saliva, causing them to emit a strong, fishy odour (Treacy *et al.* 1998). The loss of FMO3 function can also lead to problems in metabolizing tyramine (found in cheese) and some medications (Treacy *et al.* 1998).

Similarly to chickens, dietary aspects can influence TMA metabolism in humans. Cashman *et al.* (1999) found that when healthy test subjects consumed Brussels sprouts for three weeks, the TMA to TMAO ratio of their urine increased by 2.6 to 3.2 times.

They postulated that glucosinolates present in the Brussels sprouts inhibited the action of FMO3 in the liver. Other vegetables of the *Cruciferae* family, such as cabbage, broccoli, and cauliflower, would likely produce the same effect.

2.1.3 Dairy Cows

A fishy flavour has been reported in milk from Swedish Red and White dairy cows (Lundén *et al.* 2002a and 2002b). The occurrence of fishy taint in milk is often associated with cows grazing on wheat pasture (Mehta *et al.* 1974), although it has also been reported to occur in cows that have not been grazed on wheat (Lundén *et al.* 2002a). The production of tainted milk is intermittent but specific to individual cows within a herd (Lundén *et al.* 2002a).

The fishy taint is due to high levels of TMA in the milk (Lundén *et al.* 2002a). A possible source of the TMA is bacterial degradation of lecithin, choline, and/or betaine to TMAO because TMA is an intermediate product in these reactions (Eyer *et al.* 1990).

Lundén *et al.* (2002a) found that TMA concentrations between 1 to 37 µg/g in milk resulted in a fishy taint. They also found that the degree of taint is associated with TMA concentration in a dose-dependant manner. The human detection threshold for TMA in milk is estimated to be 2 µg/g (Mehta *et al.* 1974). Pasteurization does not improve the flavour of fishy-tainted milk (Mehta *et al.* 1974), but a drop in pH will cause TMA to be converted to trimethyl ammonium, which is not volatile and therefore does not result in a fishy taste (Lundén *et al.* 2002a).

Lundén *et al.* (2002b) reported that the accumulation of TMA in milk is due to a nonsense mutation in the *FMO3* gene of affected cattle. A nucleotide substitution of C

to T at nucleotide 62 of exon 6 causes a premature stop codon at amino acid position 283, rendering the FMO3 enzyme non-functional. It has been found in Swedish Red and White cattle, but not in the other dairy breeds tested, including Holstein, Jersey, and Swedish Polled. Lundén *et al.* (2002b) hypothesized that milk taint is inherited recessively but heterozygotes can express the phenotype under certain environmental conditions.

2.2 Canola Meal and Rapeseed Meal

Rapeseed (*Brassica napus* and *B. campestris*) is a member of the mustard family, which also includes cabbage, turnips, Brussels sprouts, and several other vegetables (Shahidi 1990). Rapeseed oil was used for centuries in Europe and Asia for soap making, lamp oil, and occasionally human consumption. After the advent of the industrial revolution it was used as an industrial lubricant (National Research Council 1992). Prior to World War I rapeseed meal was used primarily as a fertilizer, then later at low concentrations in livestock diets (National Research Council 1992).

Rapeseed is well adapted for growth on the Canadian prairies and has a relatively high oil content; these attributes made it an attractive candidate for the development of a new edible oil source after World War II (National Research Council 1992). Rapeseed contains erucic acid (22 to 60% of the oil) and glucosinolates (0.30 to 0.63% of the meal), both of which are toxic and make the oil and meal unpalatable (Shahidi 1990, National Research Council 1992).

In the 1950's an intensive breeding and selection program began in Canada to make rapeseed an appealing edible oil source (National Research Council 1992). In 1968 the first low erucic acid (less than 5%) variety was released and in 1974 the first

"double low" or low glucosinolate low erucic acid variety was licensed. In 1979 all varieties in Canada containing less than 2% erucic acid and 30 μmol/g of glucosinolates were given the name "canola". Canola and rapeseed were officially recognized as different species by the US Food and Drug Administration in 1985, and canola was given the status as generally regarded as safe (Shahidi 1990). Canola oil is now a popular edible oil and canola meal is commonly used as a protein supplement in livestock rations (National Research Council 1992).

2.2.1 Factors Affecting Fishy Egg Taint

Feeding rapeseed meal (RSM) to brown-shell laying strains of hens has been reported in many instances to cause a fishy taint in some eggs (Hobson-Frohock *et al.* 1975, Overfield and Elson 1975, Marangos and Hill 1976, Pearson *et al.* 1979). Overfield and Elson (1975) found that feeding RSM, even at levels as low as 3%, resulted in the production of fishy-tainted eggs from susceptible hens. The fishy eggs appeared within 5 days of incorporating RSM into the diet. Removal of RSM from the diet stopped the production of tainted eggs. Egg tainting is still a problem with modern-day canola meal, as Perez-Maldonado and Barram (2004) found that diets of 15 and 20% canola meal led to the production of fishy-tainted eggs when feed to brown-shelled layers.

The incidence of tainting can decrease over time. Overfield and Elson (1975) found that when RSM was fed for an extended period of time, the percentage of eggs that were tainted decreased substantially after 3 weeks on trial from 20 to 11%. They theorized that the hens were able to adapt to the dietary factor that was causing the

production of tainted eggs. They even went as far as to suggest that pullets reared on a diet containing RSM would not produce tainted eggs as adults. March and MacMillan (1979) also found that the incidence of tainted eggs decreased substantially over time (27 weeks) when hens were maintained on a diet containing elevated levels of choline. They postulated that the hens might adapt to the presence of elevated choline levels in the diet. Further study showed that this was not the case and they concluded that their original observations were due to the fact that taint-producing hens do not lay tainted eggs consistently. They proposed that the inconsistency of tainted egg production could be due to shifts in the bacterial population of the small intestine and ceca.

Rapeseed meal and canola meal (CM) inhibit the ability of some hens to oxidize TMA into TMAO. Pearson *et al.* (1979) found that feeding RSM to taint-producing hens substantially reduced their ability to convert ^{14}C-TMA into ^{14}C-TMAO. Tannins from RSM have been shown to reduce the ability of hens to oxidize TMA by up to 32% (Fenwick 1981). The tannin content of low and high glucosinolate varieties of RSM is similar. Dehulling rapeseed before processing into RSM would substantially decrease the tannin content of the meal, but the extra cost involved is not economically feasible (Fenwick 1981).

Washburn (1990) states that although RSM is the "primary dietary factor influencing the production of tainted eggs, it is not the only one since the incidence in any individual bird is variable." Since RSM contains very little free TMA, it must contain a precursor to TMA in order to increase the circulating TMA levels in affected birds (Hobson-Frohock *et al.* 1975).

2.2.1.1 Glucosinolates

Glucosinolates, also known as thioglucosides, are a type of antinutritional factor found in RSM (Bell and Belzile 1965) as well as canola meal, though at levels below 30 µmol/g (Hickling 2001). The major glucosinolate found in RSM and canola meal (CM) is sometimes called progoitrin because, it is converted to goitrin by the enzyme myrosinase. Goitrin is a goitrogenic compound and its chemical name is (-)-5-vinyl-2-oxazolidinethione (Bell and Belzile 1965).

Myrosinase, also known as thioglucosidase, cleaves glucosinolates to yield isothiocyanates (specifically 3-butenyl and 4-pentenyl isothiocyanate in the case of RSM) as well as bisulfate and glucose (Bell and Belzile 1965). Myrosinase and glucosinolates are separated in intact rapeseeds but are able to combine when the seed is processed to form RSM (Josefsson 1972). Myrosinase is also produced by enteric bacteria (Oginsky *et al.* 1965). Goitrin is not a primary product of myrosinase activity. It is formed when isothiocyanates cyclize (Figure 2.2, Bell and Belzile 1965). Goitrin formation is favored by heat treatment and extended storage (Josefsson 1972).

Goitrin (from glucosinolates) inhibits the oxidation of TMA to TMAO (Goh *et al.* 1983, Goh *et al.* 1985). Pearson *et al.* (1983) found that RSM from a low glucosinolate variety decreased hepatic TMA oxidase activity by 34%, but a high glucosinolate variety decreased activity by 77%. The addition of glucosinolates to the low glucosinolate RSM diets yielded results similar to those of the high glucosinolate RSM diets. It was concluded that the glucosinolates present in RSM depress hepatic TMA oxidase (*i.e.* FMO3) activity (Pearson *et al*. 1983, Goh *et al.* 1985). Goitrin

inhibits the action of TMA oxidation by competing with TMA for the active site on FMO3 (Fenwick *et al.* 1981).

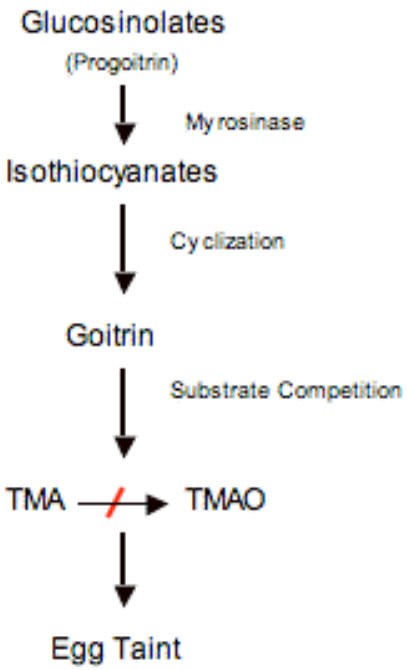

Figure 2.2 Schematic representation of the involvement of glucosinolates in the development of fishy-egg taint. Goitrin inhibits the oxidation of trimethylamine (TMA) to trimethylamine *N*-oxide (TMAO).

RSM can increase thyroid size when fed to laying hens at concentrations above 10% (Marangos and Hill 1976, Pearson *et al*. 1978, Ibrahim and Hill 1980). Due to the goitrogenic properties of RSM, it was postulated that the thyroid gland might be involved in egg tainting. Fenwick *et al.* (1981) found that including goitrin or a synthetic goitrogen in the diet of affected hens had the same effect as feeding RSM on depressing the conversion of ^{14}C-TMA to ^{14}C-TMAO. They also found that injecting thyroid

hormones did not improve ^{14}C-TMA metabolism. They therefore concluded that the thyroid is not involved in fishy-egg taint.

Pearson *et al.* (1983) found that susceptible hens still produced tainted eggs when fed a low glucosinolate variety of RSM. They concluded that reducing the glucosinolate content of rapeseed alone would not be effective in eliminating egg tainting, and that another factor must be present in CM that leads to the production of fish-tainted eggs.

2.2.1.2 Sinapine and Choline

Sinapine is an ester of choline and 4-hydroxy-3, 5-dimethoxy-cinnamic acid, also known as sinapic acid (Figure 2.3, Pearson *et al.* 1980). It is the major form of choline found in canola meal, present at approximately 1 to 2% (Lacki and Duvnjak 1996). Sinapine is hydrolyzed in the hindgut by enteric bacteria to yield choline and sinapic acid (Qiao and Classen 2003). Qiao and Classen (2003) found that the apparent ileal digestibility of sinapine from RSM was 30-46%, whereas the fecal digestibility was 54-63%, suggesting that the ceca are the major site of hydrolysis to choline and sinapic acid.

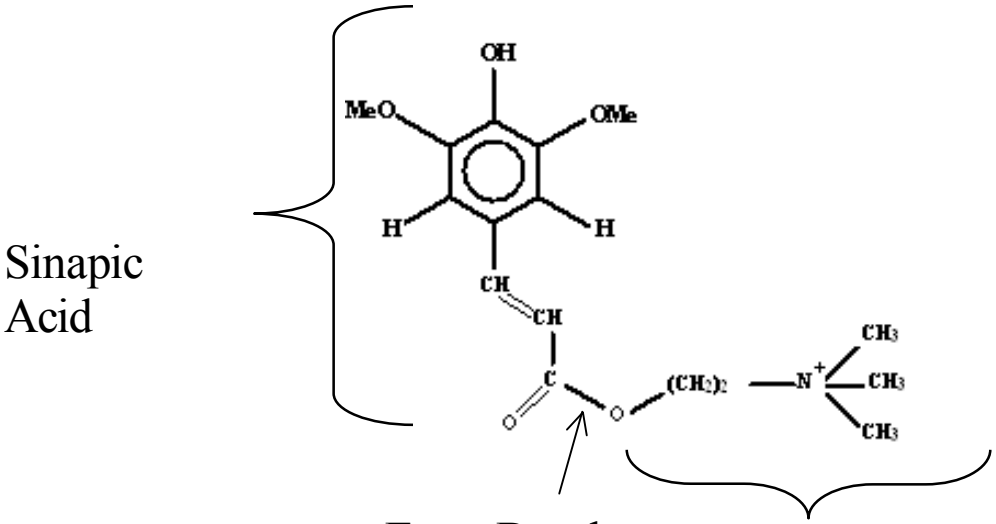

Figure 2.3 Diagrammatic representation of sinapine (sinapic acid and choline joined by an ester bond). Modified from Mabon *et al*. 1999.

Decreases in the dietary level of sinapine led to decreases in the TMA content of eggs laid by tainting hens (Hobson-Frohock *et al.* 1977). Goh *et al.* (1979a) found that including more than 0.1% sinapine in a RSM based diet led to the production of tainted eggs by susceptible hens. They also found that the higher the level of sinapine in the diet, the less time it took for TMA to reach its maximum level in the eggs. The sinapine content of RSM varies significantly with cultivar, therefore it may be possible to select for and develop a low sinapine variety of RSM (Mueller *et al.* 1978, Zum Felde *et al.* 2007).

Goh *et al.* (1979b) found that adding choline chloride to a laying diet did not lead to the production of tainted eggs, but adding bound choline in the form of sinapine did lead to the production of tainted eggs from susceptible hens. They postulated that this was because choline chloride was readily absorbed by the small intestine and therefore was not available to TMA-producing bacteria in the ceca. If the bacteria do not have access to the choline they cannot convert it to TMA and subsequently there is little or no TMA to absorb and be deposited in the eggs.

According to March and MacMillan (1980), over 50% of the choline present in RSM is in a bound form. They also found that on average the choline content of RSM was 5140 mg/kg compared to 1850 mg/kg in soybean meal. Dänicke *et al.* (2006) found a significant relationship between the choline level of the diet and the occurrence of tainted eggs.

The choline requirement of laying hens is small, as they are able to synthesize large amounts of choline on their own (Crawford *et al.* 1969, Nesheim *et al.* 1971). Laying hens require approximately 120 mg of dietary choline per day for choline intake to equal output (Crawford *et al.* 1969). A recent study by Dänicke *et al.* (2006) sets the minimum choline requirement for optimum laying performance at 1500 mg/kg of feed. It may be possible to decrease the amount of supplemental choline added to the diet (to reduce the potential for fishy taint) without impacting production parameters.

2.2.1.2.1 Fermentation to Trimethylamine

Enteric bacteria hydrolyze TMA from choline through the action of bacterial deaminase (Figure 2.4, March and MacMillan 1979, Butler and Fenwick 1984). Gut bacteria convert choline exclusively to TMA, not other N-containing compounds (de la Huerga and Popper 1952).

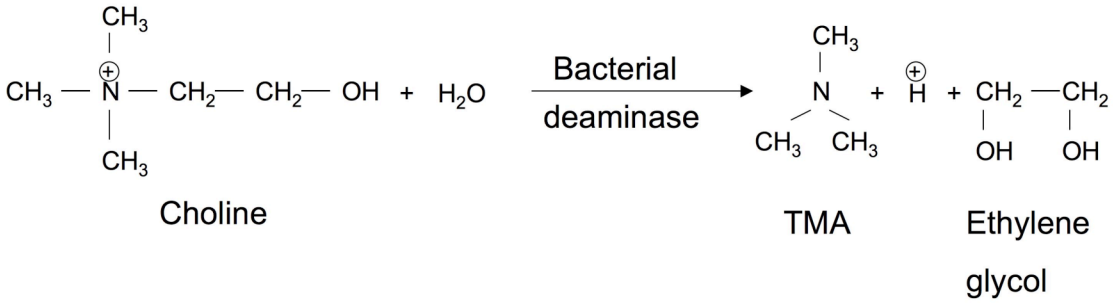

Figure 2.4 Diagrammatic representation of the hydrolysis of choline to trimethylamine (TMA) and ethylene glycol by bacterial deaminase. Redrawn from Butler and Fenwick 1984.

March and MacMillan (1979) found that the contents of the small intestine and ceca of both tainting and non-tainting hens consistently contained TMA. Concentrations of TMA were higher in the ceca than in the small intestine. TMA concentrations in the

ceca were higher when the hens were fed diets containing RSM or supplementary choline, whereas levels in the small intestine were less influenced by the diet. There was no association between the levels of TMA in the gut and TMA in the eggs. However, Dänicke *et al.* (2006) found a linear relationship between the level of TMA in the excreta and in the egg yolk of hens fed excess amounts of choline.

Adding antibiotics, such as neomycin and tetracycline, to the diet has been shown to reduce the incidence of egg tainting, suggesting that gram-positive enteric bacteria play a significant role in the production of TMA (Zentek and Kamphues 2002). Similar results have been found when aureomycin was administered orally to humans, though the inhibition of TMA production declined within a week of continuous administration and TMA levels returned to normal after two weeks (de la Huerga *et al.* 1953). No effect was seen when the antibiotic was administered intravenously.

2.3 The Flavin-containing Monooxygenase Family

The enzyme flavin-containing monooxygenase (FMO) was first reported by Ziegler and Mitchell in 1972. At that time it was given the name microsomal mixed-function amine oxidase, however in 1974 it was renamed to microsomal flavoprotein mixed-function oxidase when it was discovered that its substrates were not limited to nitrogenous compounds (Poulsen *et al.* 1974). This enzyme was isolated from pig and human liver microsomes and was found to catalyze the *N*- and *S*-oxidation of a wide range of substrates. It was present in much higher concentrations in the liver than in any other tissue (Ziegler and Poulsen 1978). In 1984, it was discovered by Williams *et al.* that rabbit lung and liver contained distinctly different forms of FMO.

As more forms of FMO were discovered and found to be present in various tissues, the need developed for a standardized system of naming members of the FMO family. Lawton *et al.* (1994) proposed a standard system of nomenclature for mammalian FMOs. The flavin-containing monooxygenase gene family was termed FMO and each form was numbered sequentially in Arabic numerals (i.e. FMO1 through FMO6). When referring to genes or mRNA the name is italicized (i.e. *FMO1*) and when referring to the protein the name is unitalicized (*i.e*. FMO1). Hernandez *et al.* (2004) discovered several pseudo genes and proposed that they should have a lowercase 'p' suffix (i.e. *FMO7p*) to differentiate them from functional genes. No further work has been published on FMO pseudogenes, so it is not clear if the proposal by Hernandez *et al.* will become convention.

2.3.1 Gene Family

There are currently six recognized members of the *FMO* gene family, *FMO1*, *FMO2*, *FMO3*, *FOM4*, *FMO5*, and *FMO6*. *FMO*s *1* through *5* give rise to functional proteins and *FMO6* is a pseudogene (Hines *et al.* 2002). Hernandez *et al.* (2004) documented an additional five pseudogenes in humans and named them *FMP7p* to *FMO11p*.

In humans, *FMO1* through *FMO4* and *FMO6* are located in a gene cluster on chromosome 1 in a region between 1q23 and 1q25 that spans approximately 2.2 Mbp (Figure 2.5, Shephard *et al.* 1993, McCombie *et al.* 1996, Hernandez *et al.* 2004). *FMO5* is located further upstream at 1q21.1 (McCombie *et al.* 1996).

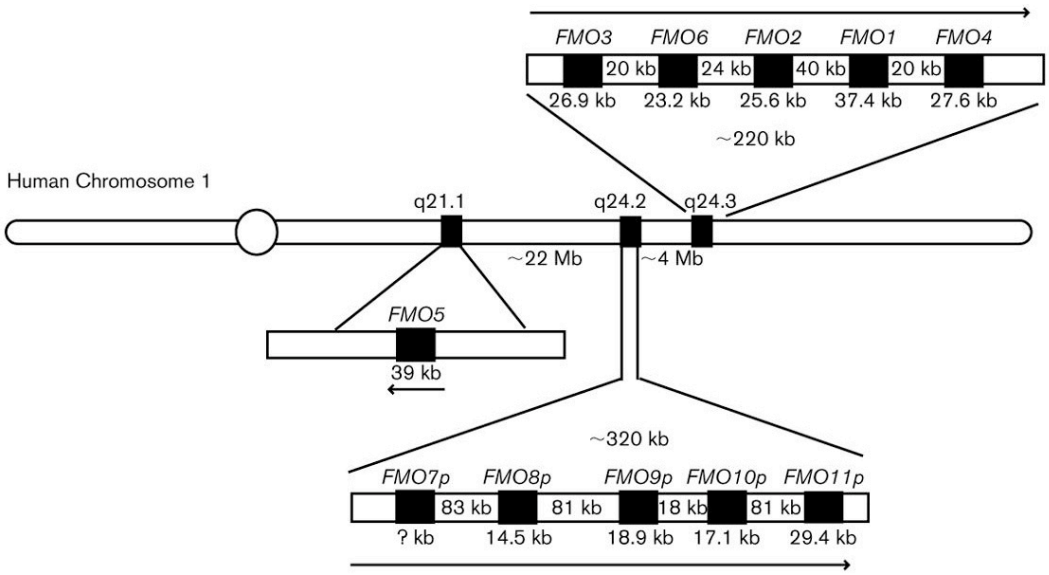

Figure 2.5 Representation of the FMO gene clusters on human chromosome 1. Taken from Hernandez *et al.* 2004.

The five functional *FMO*s (*1* through *5*) are very similar in gene structure; they all contain one or two 5' non-coding exons and eight coding exons (Figure 2.6, Hernandez *et al.* 2004). The size and intron/exon boundaries of the coding exons are highly conserved between the *FMO* genes (Hernandez *et al.* 2004).

Expression of each *FMO* is tissue, developmental, species, and in some species (such as mice and rats) sex specific (Tynes and Philpot 1987, Cherrington *et al.* 1998, Koukouritaki *et al.* 2002). Overby *et al.* (1997) found no correlation between the quantity of *FMO* mRNA and protein levels in humans, though others have found that transcription is a major factor in regulating FMO protein expression (Koukouritaki *et al.* 2002, Zhang and Cashman 2006).

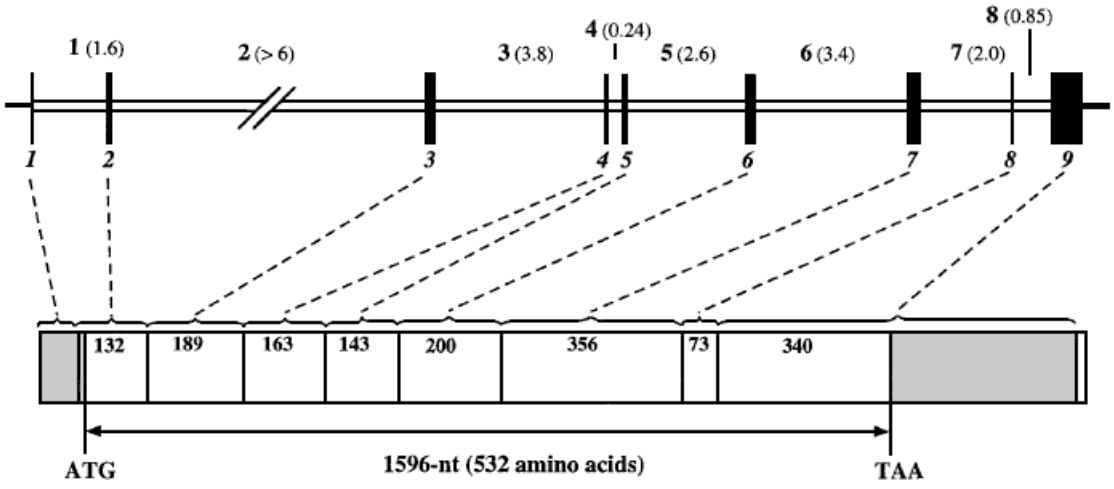

Figure 2.6 Structural representation of human *FMO3*. Exons are represented by solid boxes and italicized numbers and introns by open boxes and bolded numbers in the upper portion of the figure. The numbers in parentheses indicate the approximate sizes of the introns in kilobases. The transcribed mRNA is depicted in the lower portion of the figure. Shaded areas indicate untranslated regions and numbers indicate the nucleotide length of the coding portion of each exon. Modified from Dolphin *et al.* 1997b.

2.3.2 Structure and Function

Flavin-containing monooxygenases (FMOs) catalyze the oxidation (and thus detoxification) of a wide range of soft nucleophiles, generally nitrogen of sulfur containing organic compounds (Ziegler and Poulson 1978). Neutral compounds or those with a single positive charge are most likely to be substrates (Krueger and Williams 2005). These substrates include many xenobiotic toxins and pharmaceuticals (Ziegler 1990). Oxidation of these substrates generally reduces their toxicity and/or pharmacological activity (Krueger and Williams 2005).

The crystalline structure of a yeast (*Schizosaccharomyces pombe*) FMO was reported by Eswaramoorthy *et al.* in 2006 (Figure 2.7). The enzyme is composed of a

small and large structural domain separated by a channel. The domains are joined by a large, randomly coiled loop, which is thought to stabilize the tertiary structure. The large domain contains the FMO identifying sequence (FXGXXXHXXXF). The flavin-component, in the form of FAD, is present in the channel between the small and large domain. The adenine of FAD forms hydrogen bonds with the GAGPSG motif (FAD binding sequence) that is present at the core of the large domain. Three GXGXXG motifs also act to stabilize the FAD. Similarly, the adenine of NADPH binds to the small domain via the GGASSA motif. This bond is much weaker than the FAD bond. The flavin and nicotinamide bases form hydrogen bonds and interact with each other as part of the catalytic reaction of the enzyme (Eswaramoorthy *et al.* 2006). The enzyme also contains a highly conserved FATGY motif, which is thought to be involved in substrate recognition (Stehr *et al.* 1998).

In the first step of the catalytic reaction, $NADPH+H^+$ is bound by the enzyme, which reduces the flavin-component (FAD) to $FADH_2$ (Figure 2.7, Beaty and Ballou 1981a and 1981b, Krueger and Williams 2005). Oxygen then reacts with $FADH_2$ to form flavin-hydroperoxide (FAD-OOH). This form of FMO is stable and is how FMOs exist in the cell. The enzyme is now "activated" and as soon as a substrate containing a soft nucleophile comes into contact with the FAD-OOH it is oxidized, no substrate binding is required. The remaining hydroxyl group is converted to water and the $NADP^+$ is released. This is the rate-limiting step of the reaction. The FAD is then free to be reduced and start the cycle over again.

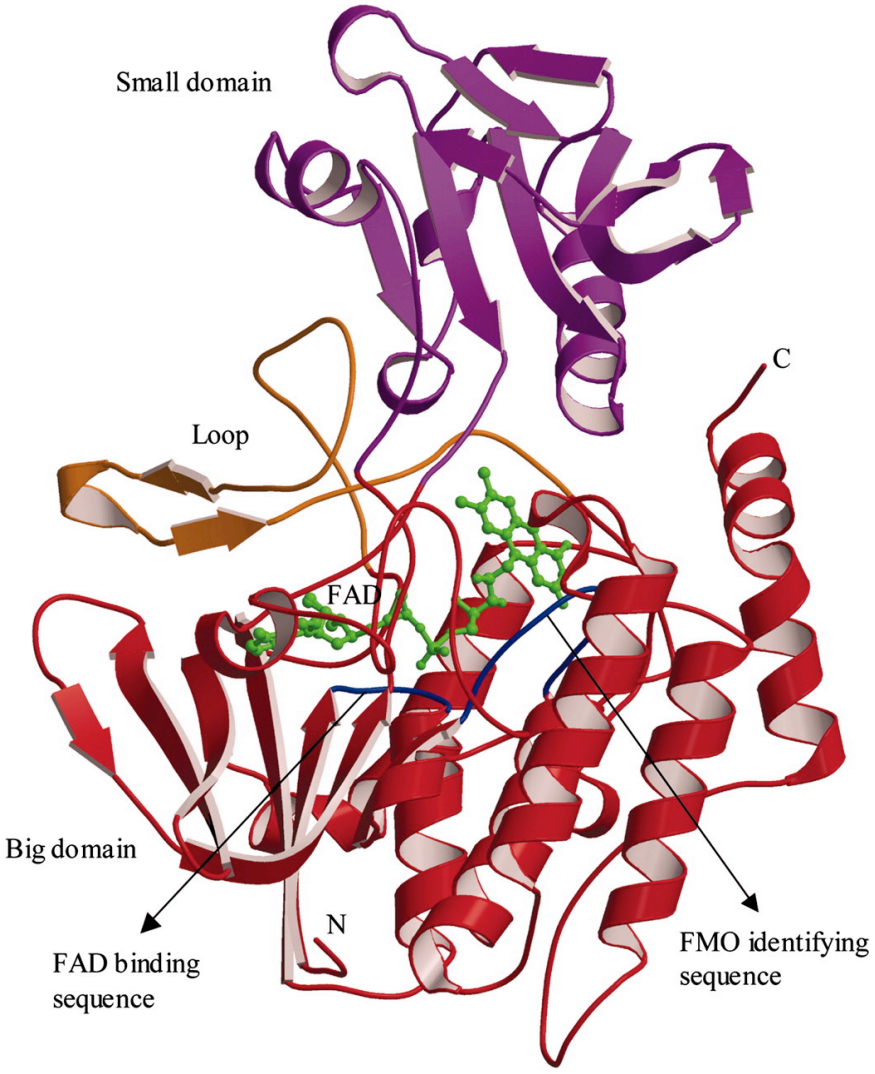

Figure 2.7 Ribbon model of *Schizosaccharomyces pombe* flavin-containing monooxygenase bound to ball and stick model of FAD (Eswaramoorthy *et al.* 2006). The small and large domains are indicated, as well as the loop that joins them. The FMO identifying sequence and FAD binding sequence are highlighted in blue.

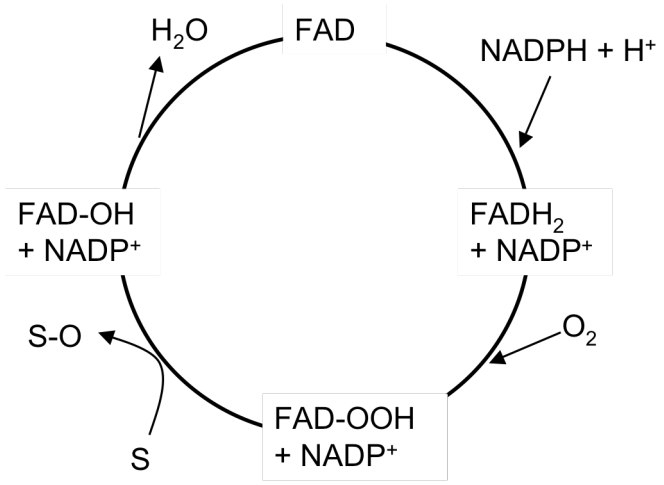

Figure 2.8 Diagrammatic representation of the oxidative mechanism of flavin-containing monooxygenase, where S is the substrate before and SO is the substrate after oxidation. Adapted from Kruger and Williams 2005.

2.3.3 FMO3

FMO3 is the enzyme responsible for the oxidation of TMA to TMAO (Lang *et al.* 1998, Zhang *et al.* 2007). The oxidation of TMA and other nucleophilic substrates by FMO3 makes them polar, which subsequently allows them to be removed from circulation by the renal system (Cashman *et al.* 2000). FMO3 is present in several tissues in the chicken, but by far the highest concentration is found in the liver (Honkatukia *et al.* 2005).

2.3.3.1 Genetics of Egg Tainting

The laying of fishy tainted eggs was found to be an individual hen characteristic (Vondell 1932). Bolton *et al.* (1976) postulated that this was a genetic condition and that it was an autosomal semi-dominant (*i.e.* additive) gene with variable expression dependent upon environmental factors. Pearson and Butler (1979) found that the TMA oxidation defect was present in both male and female chicks and there was no difference

between the sexes in regards to their sensitivity to rapeseed meal. Although fishy-egg tainting is predominantly associated with brown-shelled strains, it has been found to occur in Brown Leghorns (a white-shelled breed) and is therefore not linked to egg shell colour (Butler *et al.* 1984).

FMO3 is the only known enzyme to oxidize TMA (Lang *et al.* 1998) and mutations in *FMO3* had been reported to cause fishy-odour syndrome in humans (Dolphin *et al.* 1997a), and consequently it was a strong candidate gene for fishy-egg tainting in laying hens. Honkatukia *et al.* (2005) mapped fishy-egg tainting and *FMO3* to a region on chicken chromosome 8. *FMO3* contains one 5' non-coding exon and eight coding exons (Figure 2.9). The causative mutation for fishy-egg taint was found to be to be an A to T change at position 984 of the coding sequence, which leads to a threonine to serine amino acid substitution at position 329 in the FMO3 enzyme. The mutation will be referred to as *FMO3 c.984A>T*. This substitution occurs in the highly conserved FA**T**GY amino acid motif (Honkatukia *et al.* 2005). The mutation renders the FMO3 enzyme unable to oxidize TMA to TMAO, leading to fishy-taint syndrome in affected hens (Honkatukia *et al.* 2005).

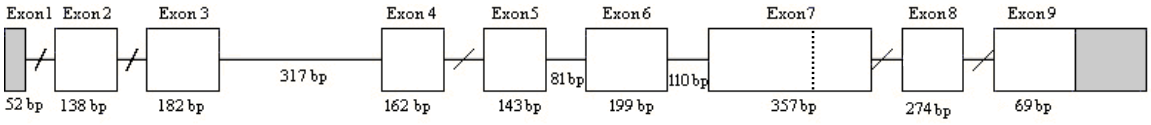

Figure 2.9 Schematic diagram of *FMO3* in *Gallus gallus*. The approximate location of the *c.984 A>T* SNP is indicated by the dashed vertical line. Modified from Honkatukia *et al*. 2005.

Honkatukia *et al.* (2005) found the *FMO3c.984A>T* mutation in several brown-shelled breeds of chickens (including three commercial lines, Marans, Green-legged partridge, and Transylvanian naked neck). They speculated that there was a common origin for the mutation, likely in the Rhode Island Red breed history. The authors found no differences in *FMO3* expression between the genotypes; therefore egg tainting is likely caused by decreased biological activity of the enzyme. Qualitatively, fishy-smelling phenotype (following dietary challenge with choline chloride) was found to be recessively inherited (Honkaukia *et al.* 2005); however Kretzschmar *et al.* (2007) found a significant difference between the yolk TMA concentrations of AA and AT hens, suggesting that egg tainting is quantitatively additive.

2.4 Objectives and Hypothesis

The purpose of this thesis was to determine the mode of inheritance of fishy-egg tainting in brown-shelled laying hens as well as to examine the effects that different sources and levels of choline in the diet have on egg tainting. We also wanted to determine if serum TMA concentration could be used to predict egg-tainting potential.

Our first hypothesis was that egg tainting is inherited through *FMO3 c.984A>T* and expression is dependent upon inclusion of TMA precursors (*i.e.* choline) in the diet. When hens are fed industry-typical levels of canola meal egg tainting is expressed additively. Secondly, we hypothesized that feeding choline in the form of choline chloride would result in lower yolk TMA concentration than choline in the form of sinapine because choline chloride is absorbed higher in the digestive tract than choline from sinapine. Lastly, we hypothesized that serum TMA concentration is linearly correlated to yolk TMA concentration.

3 MATERIALS AND METHODS

3.1 Animal Trials

Two separate feeding trials, henceforth referred to as the first-generation trial and the second-generation trial, were performed at the University of Saskatchewan Poultry Centre. A third experiment was also performed to examine the allelic and genotypic ratios of *FMO3 c.984A>T* in two commercial brown and one white-shelled strains. The experimental protocols for all three trials were approved by the Animal Care Committee at the University of Saskatchewan under the animal care protocol number 19940248.

3.1.1 First-Generation Trial

On April 27th, 2006, 600 one-day-old HyLine Brown chicks were delivered to the University of Saskatchewan Poultry Centre. There were approximately equal numbers of male and female chicks. Upon arrival each chick was fitted with a wing band embossed with a unique identification number. The chicks were dubbed (a portion of the comb was removed) and the tissue was collected for DNA extraction. The chicks were reared in floor pens under standard conditions and transferred to battery cages before the start of lay.

Prior to the commencement of the feeding trial, 84 hens genotyped for *FMO3 c.984A>T* were randomly allocated to individual battery cages. Approximately 10 hens

per genotype were allocated to each of four dietary treatments of varying levels of canola meal (Table 3.1). Only hens of the AT and TT genotypes were included in this trial due to an insufficient number of AA hens (only one AA hen was present in the population).

Table 3.1 Number of hens allocated to each treatment in the first generation trial.

Diet	Genotype	
(% Canola Meal)	TT	AT
0	10	9
6	11	9
12	10	10
18	10	15
Total	41	43

The trial began when the hens were 44 weeks of age. The hens were fed experimental diets for a three-week adaptation period followed by a one-week collection period, during which three eggs were collected per hen. Whole egg and yolk weights were recorded and the yolks were analyzed for TMA concentration.

3.1.1.1 Diets

The diets used in this trial were control (0% canola meal), 6, 12, and 18% canola meal (Table 3.2). The diets were formulated to meet the requirements outlined by HyLine (2006) and NRC (1994), assuming 111 g per hen per day feed consumption. All

diets were formulated to be isocaloric and isonitrogenous (Table 3.3). Diets were mixed at the University of Saskatchewan feed mill.

Table 3.2 Experimental diets for the first-generation trial. Formulations reported on an as-fed basis. Values are percent of diet on an as-fed basis.

Ingredient	0% CM[1]	6% CM	12% CM	18% CM
Wheat	74.85	71.63	68.89	65.91
Canola Meal	0.00	6.00	12.00	18.00
Soybean Meal	12.05	8.44	4.82	1.21
Canola Oil	1.00	1.74	2.48	3.22
Dicalcium Phosphate	0.73	0.68	0.63	0.58
Limestone	10.18	10.13	10.09	10.04
NaCl	0.23	0.23	0.23	0.23
Choline Chloride	0.10	0.10	0.10	0.10
Vit/ Min Premix[1]	0.50	0.50	0.50	0.50
Wheat Enzyme[2]	0.05	0.05	0.05	0.05
DL-Methionine	0.15	0.14	0.12	0.11
L-Lysine HCL	0.11	0.09	0.07	0.05
L-Threonine	0.04	0.03	0.01	0.00

[1]Supplied per kilogram of diet: vitamin A, 8,000 IU; vitamin D_3, 3,000 IU; vitamin E, 25 IU; menadione, 1.5 mg; riboflavin, 5 mg; pantothenic acid, 8 mg, vitamin B_{12}, 0.012 mg; pyridoxine, 1.5 mg; thiamine, 1.5 mg; folic acid, 0.5 mg; niacin, 30 mg; biotin, 0.06 mg; iodine, 0.8 mg; copper, 10 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 80 mg; zinc, 80 mg; quinguard M6S, 0.625 mg, calcium carbonate, 500 mg.
[2]Avizyme 1302. Danisco Animal Nutrition, Malborough, UK.

Table 3.3 Calculated nutrient composition of the first-generation diets. Values are listed as percent of diet on an as-fed basis, unless otherwise indicated.

Nutrient	0% CM	6% CM	12% CM	18% CM
AME[1] (kcal/kg)	2.771	2.771	2.771	2.771
Crude protein	15.50	15.50	15.50	15.50
Choline	0.122	0.150	0.178	0.207
Calcium	4.10	4.10	4.10	4.10
Chloride	0.21	0.21	0.20	0.20
Non-phytate P[2]	0.33	0.33	0.33	0.33
Total P[2]	0.49	0.52	0.54	0.57
Potassium	0.71	0.69	0.67	0.65
Sodium	0.17	0.17	0.17	0.17
Linoleic acid	1.06	1.18	1.29	1.41
Arginine	0.93	0.92	0.91	0.90
Lysine	0.80	0.80	0.80	0.80
Methionine	0.37	0.37	0.37	0.37
Met + Cys[3]	0.61	0.61	0.62	0.62
Threonine	0.56	0.57	0.58	0.59
Tryptophan	0.22	0.22	0.23	0.23

[1]Apparent metabolizable energy
[2]Phosphorous
[3]Methionine and cystine

3.1.1.2 Statistical Analysis

The experiment was a two by four factorial design, with genotype as factor 1 and diet as factor 2, and individual hens were the experimental unit. Statistical analysis was performed using the mixed procedure of SAS version 9.1 with $P<0.05$ considered significant. Means were separated using the least significant difference (LSD) method. The correlation and regression procedures of SAS were also used to examine the relationship between dietary canola meal and yolk TMA level for each of the two genotypes.

Day to day variation in yolk TMA concentration was analyzed by calculating the coefficient of variation (CV) between the three eggs analyzed for each hen and then analyzing the CVs using the mixed procedure of SAS.

3.1.2 Second-Generation Trial

Randomly selected heterozygous (AT) birds from the first generation were bred to generate birds of all three genotypes for the second trial. This was done to ensure that sufficient numbers of hens of each genotype were available for use in the trial. A total of 16 roosters were bred to 96 hens via artificial insemination. Several roosters were unable to produce sufficient semen of good quality, and were subsequently replaced with TT roosters.

Hens were inseminated with freshly collected semen once a week for four weeks. Eggs were collected daily and stored at 10 ºC until they were incubated. The eggs were candled after 10 days of incubation and transferred to the hatcher on April 31st. Chicks

were hatched, wing banded, dubbed, and placed in floor pens for rearing. Pullets were transferred to battery cages before the start of lay.

An experiment was designed to investigate the effects of *FMO3 c.984A>T* genotype and choline level and source on egg and serum TMA. Six hens per genotype (AA, AT, and TT) were assigned to one of five dietary treatments consisting of graded levels of canola meal. Additional groups of six TT hens were assigned one of four diets containing graded levels of choline chloride. A total of 114 hens were used. Only TT hens were used for the choline chloride diets because results from the first-generation trial showed egg tainting to be recessive, therefore it is unlikely that AA and AT hens would show a response to increasing levels of choline chloride.

Hens were fed the diets *ad libitum* for a three-week adaptation period and a one-week collection period. Initial weight, final weight, and egg production were recorded for each hen, and feed consumption was recorded for each diet. Three eggs were collected per hen. On the last day of the trial blood was also collected. Whole egg and yolk weights were recorded. The yolks and serum were analyzed for TMA concentration.

3.1.2.1 Diets

Commencing at 43 weeks of age, the hens were fed one of nine experimental diets for approximately four weeks. The diets (Table 3.4) consisted of a control (0.130% total choline) and four graded levels of choline total (0.158, 0.187, 0.215, and 0.243%) each from choline chloride or canola meal, for a total of nine diets. The choline present in the control diet was from the choline naturally found in the soybean meal and wheat

present in the diet. The diets were formulated to meet or exceed HyLine (2006) and NRC (1994) requirements assuming 111 g per hen per day feed intake. All diets were formulated to be isocaloric and isonitrogenous. Diets were mixed at the University of Saskatchewan feed lot.

Table 3.4 Dietary formulations for the second-generation trial. Values reported as percent of diet on an as-fed basis.

		Canola Meal (% choline)				Choline Chloride (% choline)			
Ingredient (%)	Control	0.158	0.187	0.215	0.243	0.158	0.187	0.215	0.243
Wheat	69.74	66.79	63.87	60.96	58.03	69.685	69.63	69.575	69.52
Canola Meal	0.00	6.00	12.00	18.00	24.00	0.00	0.00	0.00	0.00
Soybean Meal	15.20	11.51	7.81	4.11	0.41	15.20	15.20	15.20	15.20
Canola Oil	1.76	2.49	3.22	3.95	4.68	1.76	1.76	1.76	1.76
Celite[1]	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Dicalcium Phosphate	0.71	0.67	0.62	0.57	0.53	0.71	0.71	0.71	0.71
Limestone	10.17	10.13	10.08	10.03	9.99	10.17	10.17	10.17	10.17
NaCl	0.24	0.24	0.24	0.23	0.23	0.24	0.24	0.24	0.24
Choline Chloride	0.00	0.00	0.00	0.00	0.00	0.055	0.11	0.165	0.220
Vit/Min Premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Wheat Enzyme[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
DL-Methionine	0.13	0.12	0.11	0.10	0.08	0.13	0.13	0.13	0.13

[1]Added as an indigestible marker.
[2]Supplied per kilogram of diet: vitamin A, 8,000 IU; vitamin D_3, 3,000 IU; vitamin E, 25 IU; menadione, 1.5 mg; riboflavin, 5 mg; pantothenic acid, 8 mg, vitamin B_{12}, 0.012 mg; pyridoxine, 1.5 mg; thiamine, 1.5 mg; folic acid, 0.5 mg; niacin, 30 mg; biotin, 0.06 mg; iodine, 0.8 mg; copper, 10 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 80 mg; zinc, 80 mg; quinguard M6S, 0.625 mg, calcium carbonate, 500 mg.
[3]Avizyme 1302. Danisco Animal Nutrition, Malborough, UK.

Table 3.5 Calculated nutrient composition of the second-generation diets. All values are reported as percent of the diet on an as-fed basis, unless otherwise noted.

		Canola Meal (% choline)				Choline Chloride (% Choline)			
Nutrient	Control	0.158	0.187	0.215	0.243	0.158	0.187	0.215	0.243
AME[1] (kcal/kg)	2770	2770	2770	2770	2770	2770	2770	2770	2770
Crude protein	16.20	16.20	16.20	16.20	16.20	16.20	16.20	16.20	16.20
Choline	0.130	0.158	0.187	0.215	0.243	0.158	0.187	0.215	0.243
Calcium	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10
Chloride	0.21	0.21	0.21	0.20	0.20	0.21	0.21	0.21	0.21
Non-phytate P[2]	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33	0.33
Total P[2]	0.49	0.52	0.54	0.57	0.60	0.49	0.49	0.49	0.49
Potassium	0.76	0.74	0.72	0.70	0.68	0.76	0.76	0.76	0.76
Sodium	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Linoleic acid	1.16	1.28	1.39	1.51	1.62	1.17	1.18	1.19	1.12
Arginine	1.00	0.99	0.98	0.96	0.95	1.00	1.00	1.00	1.00
Lysine	0.78	0.79	0.81	0.82	0.83	0.78	0.78	0.78	0.78
Methionine	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Met + Cys[3]	0.61	0.62	0.62	0.63	0.63	0.61	0.61	0.61	0.61
Threonine	0.55	0.58	0.60	0.63	0.65	0.55	0.55	0.55	0.55
Tryptophan	0.23	0.24	0.24	0.24	0.24	0.23	0.23	0.23	0.23

[1]Apparent metabolizable energy.
[2]Phosphorous.
[3]Methionine and cystine.

3.1.2.2 Statistical Analysis

A five by three factorial analysis was performed to examine the effects of canola meal level and *FMO3 c.984A<T* genotype on yolk and serum TMA concentration. Analysis was performed using the mixed procedure of SAS 9.1. Significance was set at $P<0.05$ and means were separated using the LSD method. Regression analyses between canola meal level and yolk TMA concentration were performed separately for each genotype. Egg production and average daily gain were analyzed using the same procedures as above.

Day to day variation in yolk TMA concentration was analyzed by calculating the coefficient of variation (CV) between the three eggs analyzed for each hen and then analyzing the CVs using the mixed procedure of SAS. The CV was calculated as the ratio of the standard deviation to the mean.

A two by five factorial design was used to analyze the effects of choline source and concentration on yolk TMA concentration. Significance was again set at $P<0.05$, and the LSD procedure was used to separate the means. Regression analyses were performed for each of the choline sources.

3.1.3 Other Layer Strains

Genotypic frequencies for *FMO3 .984A>T* were examined in two additional brown-shelled and one white-shelled laying strains. Blood was collected from 100 Lohmann Brown, ISA Brown, and Lohmann White Leghorn hens. DNA was extracted and the hens were genotyped for the SNP.

3.2 DNA Extraction

Genomic DNA was extracted from comb tissue or blood for genetic analysis.

3.2.1 From Tissue

At one day of age each chick was dubbed to collect a sample of comb tissue. Surgical scissors were used to remove a portion of the comb. Scissors were dipped in ethanol between chicks. The collected tissue was immediately placed in a 1.5 ml microcentrifuge tube containing 300 µl of lysis buffer. The lysis buffer consisted of 9.4 mM Tris pH 8, 0.375 M NaCl, 1.9 mM disodium EDTA, and 6.25% SDS. Samples were stored at -20 ˚C until time of extraction.

Samples were thawed to room temperature and 3 µl of 20 mg/ml proteinase K was added. The tubes were gently inverted several times to mix and incubated overnight at 55 ˚C in a shaking water bath. Samples were cooled to room temperature and 100 µl of 4M ammonium acetate was added. Samples were vortexed at high speed for approximately 20 seconds to mix and then centrifuged at 13,900 RMP for 3 minutes.

The supernatant was transferred to a new 1.5 ml microcentrifuge tube containing 300 µl of isopropanol. The tubes were inverted 50 times to mix and then centrifuged for 3 minutes at 13,900 rpm. The supernatant was discarded and the tubes air-dried before 300 µl of 70% ethanol was added. The tubes were inverted gently several times to wash the DNA pellet and then centrifuged for 3 minutes at 13,900 rpm. The supernatant was again removed and the tubes were air-dried before 500 µl of pH 7.4 TE buffer was added. The DNA was allowed to resuspend into solution prior to storage at -20 ˚C.

Two modifications were made to the extraction procedure for the second generation of chicks. Firstly, the proteinase K incubation temperature was increased from 55 ˚C to 60 ˚C to optimize digestion. Secondly, the volume of TE buffer used to resuspend the DNA pellet was reduced from 500 µl to 100 µl to increase the concentration of DNA in the sample.

3.2.2 From Blood

Samples were collected via brachial veinipuncture into 4 ml EDTA vaccutainer tubes and stored at -20 ˚C. Samples were allowed to thaw overnight at 4 ˚C prior to extraction.

The procedure for extraction was modified from that of Fitzsimmons *et al.* (1998). Five hundred microlitres of lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$.1% Triton X, and 10 mM Tris pH 7.5) was added to 250 µl of blood in a 1.5 ml microcentrifuge tube. The samples were vortexed at high speeds for 10 to 20 seconds and then centrifuged at 10,000 rpm for 5 minutes.

The supernatant was discarded and 500 µl of lysis buffer was added. The samples were vortexed to resuspend the pellet and centrifuged at 10,000 rpm for 5 minutes. These steps were repeated twice, after which the supernatant was clear in colour.

The pellet was then resuspended in 500 µl of a PCR extraction buffer. The buffer consisted of 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Tween 20, and 0.45% Nonident P40. Each sample was incubated with 10 µl of 20 mg/ml proteinase K overnight at 65 ˚C in a shaking water bath. After incubation

some protein residue remained in the bottom of the tubes, so the supernatant was transferred to new 1.5 ml microcentrifuge tubes.

To precipitate the DNA, 50 µl of 3 M sodium acetate was added. The solution was mixed by inversion and approximately 1 ml of 95% ethanol was added. The samples were stored overnight at -20 ˚C and then centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and 500 µl of 70% ethanol was added. The samples were vortexed briefly and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was again discarded and the remaining DNA pellet was resuspended in 500 µl of pH 8.0 TE buffer. The samples were allowed to resuspend at 4 ˚C overnight and then stored at -20 ˚C.

3.3 Genotyping

All animals were genotyped for *FMO3 c.984A>T*, reported by Honkatukia *et al.* (2005). A Polymerase Chain Reaction-Restriction Length Polymorphism (PCR-RFLP) was developed with the aid of Sequencher version 4.1 (Genecodes) software and the reported genomic *FMO3* sequence for *Gallus gallus* (Genbank accession number AH012591).

The forward and reverse primers (forward: 5'-GCT CAT CAC CCG CTT CTG G-3'; reverse: 5'-GCC TCG TTG TTC TTG CTT TCG-3') were designed using OligoAnalyzer version 3.1 (Integrated DNA Technologies) and were obtained from Operon Biotechnologies, Inc. The primers were resuspended in 1 ml of TE buffer (pH 8.0) and stored at -20 ˚C. Aliquots were diluted to 10 pmol/µl and stored at 4 ˚C.

Each PCR reaction consisted of 1 µl of DNA template and 20 µl of PCR cocktail (0.2 pmol forward primer, 0.2 pmol reverse primer, 0.2mM dNTP mix (Fermentas), 1X

PCR buffer (Fermentas), 1.9 mM $MgCl_2$ (Fermentas), and 0.5 units of Taq polymerase (Fermentas) in ultra pure water. A drop of mineral oil was added to each tube to prevent evaporation. Thermocycling was performed using a Robocycler Gradient 96 thermocycler (Stratagene). The reaction began with a two-minute denaturation period at 94 ˚C. This was followed by 35 cycles of 50 seconds at 94 ˚C, 50 seconds at 63 ˚C, and 50 seconds at 72 ˚C. The last step was four-minute final extension period at 72 ˚C.

The resulting 462 bp PCR product was then digested with the restriction endonuclease *BsrI* (Fermentas). Directly added to each reaction product was 10 units of *BsrI* and 2 µl of Buffer B (Fermentas), which was then incubated for 4 hours at 65 ˚C. The cleavage sequence for *BsrI* is **A**CTGGN|, which subsequently cleaved the A allele of the *FMO3 c.984A>T* SNP but not the T allele. This resulted in the T allele remaining 462 bp and the A allele being cleaved into 400 and 62 bp fragments. The digest products were electrophorized on a 2% agarose gel to visualize the differences in fragment length

3.4 Trimethylamine Determination

The procedures for determination of TMA concentration in serum and egg yolk were modified from those reported by Reese *et al*. (2004).

3.4.1 In Egg Yolk

Eggs were collected on the day of lay and stored for up to one week at 4 ˚C. Whole egg weight was recorded. The yolk was separated, weighed, and stored in a 50 ml centrifuge tube at -20 ˚C. The yolk was thawed and 1 g of 10% trichloracetic acid

(TCA) in water was added per gram of yolk to precipitate protein. The yolk and acid were mixed with a metal spatula to homogenize and allowed to sit overnight at room temperature.

The next day the solution was filtered through a folded sheet of 11 cm Whatman #2 filter paper. The filtrate was stored for up to four weeks at 4 ˚C. A 2 ml aliquot was transferred to a 16 X 100 glass culture tube and 1.5 ml of potassium hydroxide (KOH) was added to separate trimethylamine from its salts. The tube was vortexed briefly and 0.5 ml of neutral buffered formalin (NBF) was added to fix the other nitrogenous compounds. The tube was again briefly vortexed and 5 ml of toluene was added. A set of 40 tubes, capped with polypropylene stoppers, was shaken at 250 rpm in an orbital shaker for 2 hours at room temperature. This allowed the free trimethylamine to move from the aqueous phase into the toluene.

After shaking, a 2.5 ml aliquot of the toluene phase was transferred to a new 16 X 100 tube and 2.5 ml of 0.02% picric acid in toluene was added. The picric acid formed a yellow picrate complex with the nitrogen of the TMA. Approximately 1.5 ml was transferred to a plastic cuvette and placed in a spectrophotometer and the absorbance at 410 nm was recorded. A standard curve was produced from 14 TMA standard solutions (ranging in concentration from 0 to 29.3 μg/ml TMA-nitrogen) to calculate the TMA-nitrogen concentration of each yolk sample. TMA concentration was then calculated from the TMA-nitrogen concentration.

3.4.2 In Serum

Blood was collected via brachial veinipuncture into 4 ml vaccutainer serum tubes. The tubes were stored overnight at 4 ˚C to encourage clotting and then centrifuged at 3000 rpm for 5 minutes to separate the serum from the clot. The serum was transferred to screw cap microcentrifuge tubes and stored at -20 ˚C until the time of analysis.

Serum was thawed at room temperature. A 650 µl aliquot of serum was mixed with 650 µl of 10% TCA in a 1.5 ml centrifuge tube. If 650 µl of serum was not available the total amount present was used and volumes of the solutions added were adjusted accordingly. The tubes were vortexed and then centrifuged at approximately 14,000 rpm for 5 minutes. A 1 ml aliquot of the supernatant added to 750 µl of KOH in a 16 X 100 culture tube and vortexed, followed by the addition of 250 µl NBF. The tube was again vortexed and 2.5 ml of toluene was added. The tube was capped and a set of 40 tubes was shaken at 250 rpm for 2 hours. In a plastic cuvette, 500 µl of the toluene supernatant was added to 500 µl of 0.02% picric acid in toluene to form a yellow TMA-nitrogen picrate complex. The spectrophotometric absorbance at 410 nm was recorded and a standard curve was used to calculate the TMA-nitrogen, and subsequently TMA, concentration of the serum sample.

3.5 Feed Analysis

Dry matter content of each diet was determined using AOAC (1990) method 930.15. Crude protein was analyzed using AOAC (1995) method 990.03.

3.5.1 Choline Determination

Free choline concentration was analyzed for each experimental diet was well as samples of the soybean meal and canola meal used in the diets. Extraction was performed using the procedures developed by Menten and Pesti (1998). In a Goldfisch beaker, 2 g of finely ground feed ingredient was combined with 25 ml of extractant (0.5 M KOH in methanol). Boiling beads were added prior to simmering for 2 hours on a Goldfish apparatus. When the solution had cooled to room temperature 30 ml of distilled water was added and the pH was adjusted to 6.0 to 6.5 with HCl. The solution was filtered through one 11 cm sheet of Whatman #2 filter paper into a 100 ml volumetric flask and the volume of the filtrate was increased to 100 ml with distilled water. This solution was then used for choline quantification.

The Choline/Acetylcholine Quantification Kit from BioVision Research Products (catalog #K615-100) was used to determine the choline concentration of the filtrate. The kit oxidizes free choline to betaine, and this reaction yields products that react with the supplied Choline Probe and fluoresce at 587 nm when excited at 535 nm. Standards and samples were prepared in a 96-well microtiter plate. The standards (50 µl each of 0, 2, 4, 6, 8, and 10 pmol/µl of choline) were prepared using the choline standard supplied in the kit. Samples were prepared by adding 1 µl of filtrate (from the extraction) to 49 µl of Choline Assay Buffer (supplied in the kit).

To each well was added 48 µl of a reaction mix, which consisted of 44 µl of Choline Assay Buffer, 2 µl of Choline Probe (dissolved in anhydrous DMSO), and 2 µl of Enzyme Mix (dissolved in Choline Assay Buffer). The fluorescence was read in a fluorometer at Ex/Em = 535/590 nm. The background reading (from the 0 pmol/µl

standard) was subtracted from all of the fluorescence readings and a standard curve was used to calculate the concentration of choline in the filtrate. Dilution factors were then used to calculate the concentration of free choline in the samples.

4 RESULTS

4.1 First-Generation Trial

4.1.1 Genotyping

A representative agarose gel of the *FMO3 c.984A>T* PCR-RFLP is presented in Figure 4.1. Representative PCR products of each genotype were sequenced by the Plant Biotechnology Institute – National Research Council (Saskatoon, Saskatchewan) to confirm the accuracy of the test. A BLAST search (National Center for Biotechnology Information) of the sequence also confirmed that only *FMO3* was being amplified.

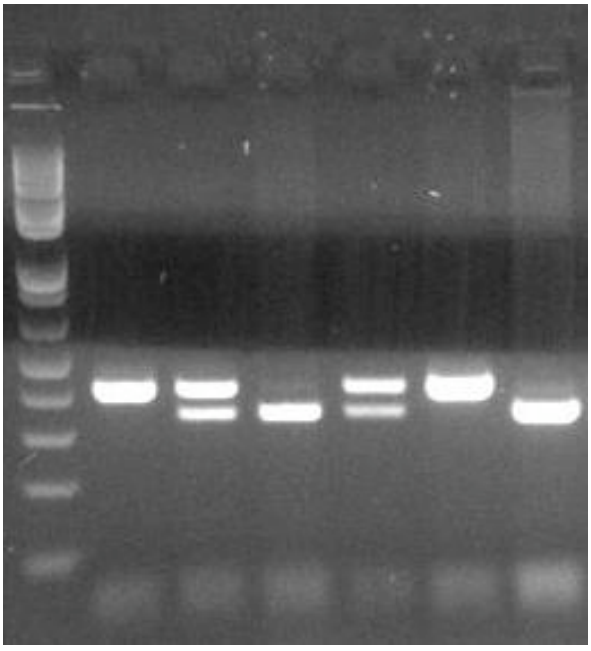

Figure 4.1 Representative gel for the *FMO3 c.984A>T* PCR-RFLP. Lane 1 is a kb1+ ladder, lanes 2 and 6 are TT, lanes 3 and 5 are AT, and lanes 4 and 7 are AA chickens respectively.

The allele frequencies of the first generation were not in Hardy-Weinberg equilibrium (Table 4.1). See Appendix A for individual genotype data

Table 4.1 First-generation genotypic and allelic frequencies for *FMO3 c.984A>T*, where n is the total number of birds per genotype.

Genotype	n	Frequency
AA	7	0.01
AT	473	0.79
TT	118	0.20
Allele		Frequency
A		0.41
T		0.59

4.1.2 Yolk TMA

4.1.2.1 Genotype and Canola Meal

Genotype, diet, and their interaction all had highly significant effects on yolk TMA concentration (Table 4.2, Figure 4.2). See Appendix B for individual yolk TMA concentrations. The mean TMA concentration of the three eggs collected was used for statistical analysis. Hens of the AT genotype did not demonstrate a response in yolk TMA concentration to increasing levels of canola meal. In contrast, there was a significant linear regression between canola meal level and yolk TMA concentration for hens of the TT genotype (

Table 4.3). The regression equation for the TT genotype is TMA = 0.304CM + 2.703, therefore the calculated maximum inclusion level of CM (to produce a yolk TMA concentration of 4 μg/g) is 4%.

Table 4.2 Results of the test of main effects on yolk TMA concentration in the first-generation trial.

Main Effect	P Value
Genotype	<0.0001
Diet	<0.0001
Interaction	<0.0001

Table 4.3 Results of the linear regression analysis between yolk TMA concentration (μg/g) and canola meal (%) in the first-generation trial, separated by genotype.

Genotype	P Value	R^2 Value
AT	0.3371	0.0243
TT	<0.0001	0.5149

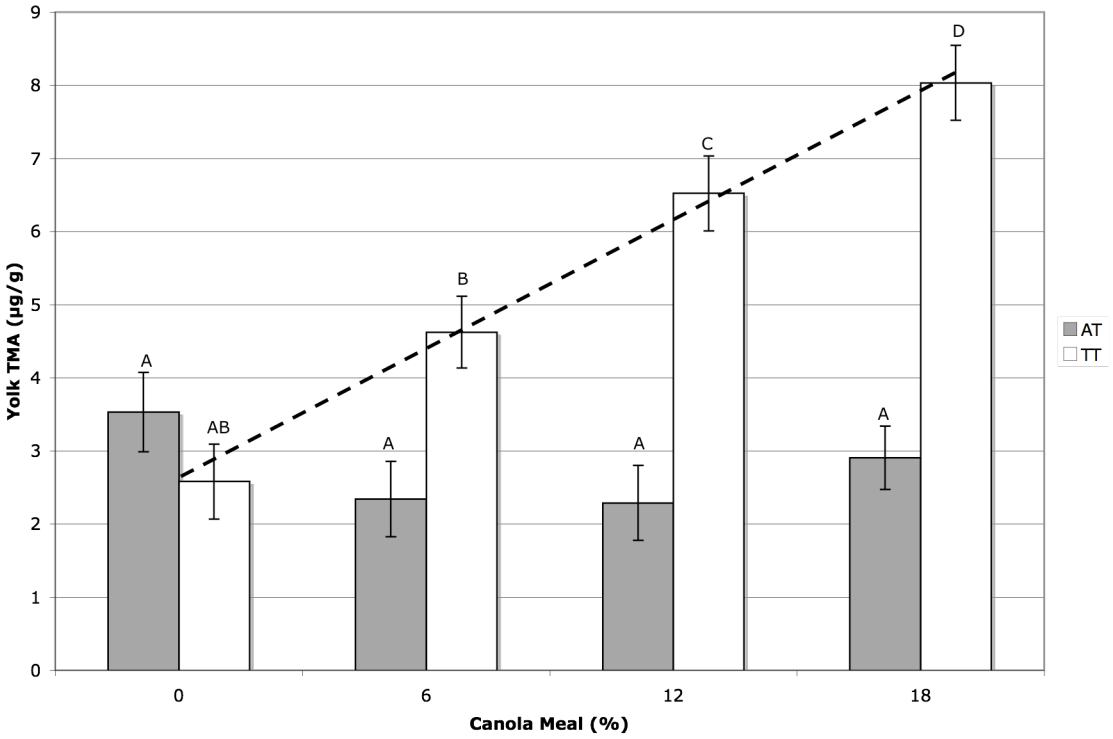

Figure 4.2 The effects of genotype and diet (% canola meal) on yolk TMA concentration (µg/g) in the first-generation trial. The dashed line represents the regression equation for the TT genotype. Means with the same letter are not significantly different ($P<0.05$). Error bars indicate the standard error of the mean.

4.1.2.2 Variation

A coefficient of variation (CV) was calculated for each hen using the yolk TMA concentrations from the three eggs collected (see Appendix B for individual CVs). Only genotype had a significant effect on the CV (Table 4.4). There was significantly greater day-to-day variation in yolk TMA concentration for AT hens than for TT hens (Table 4.5).

Table 4.4 The main effects of genotype, diet, and their interaction on the CV of yolk TMA concentration.

Main Effect	P Value
Genotype	0.0039
Diet	0.0934
Interaction	0.1999

Table 4.5 Coefficient of variation of individual yolk TMA concentration as separated by *FMO3 c.984A>T* genotype.

Genotype	AT	TT
Coefficient of Variation	48.52^{A}	34.27^{B}
SEM	3.43	3.32

^{AB}Means in the same row that do not share a common superscript are significantly different ($P<0.05$). SEM=standard error of the mean.

4.2 Second-Generation Trial: Genotype and Canola Meal Level

In this section, the results of the *FMO3* genotype (AA, AT, or TT) by canola meal level (0, 6, 12, 18, or 24%) trial are presented. The results of the choline chloride diets are presented in section 4.3.

4.2.1 Genotyping

The genotypic ratios in the second generation differ slightly from the expected 1:2:1 ratio of heterozygote to heterozygote mating because three AT roosters were

replaced by TT roosters because of poor sperm quality (Table 4.6). See Appendix C for individual genotypes.

Table 4.6 Second-generation genotypic and allelic frequencies for *FMO3 c.984A>T*, where n is the total number of birds per genotype.

Genotype	n	Frequency
AA	163	0.20
AT	398	0.50
TT	241	0.30
Allele		Frequency
A		0.45
T		0.55

4.2.2 Yolk TMA

4.2.2.1 Genotype and Canola Meal

The effects of genotype, diet, and their interaction on yolk TMA concentration were all significant (Table 4.7). There was no significant response in yolk TMA concentration from hens of the AA and AT genotypes with increasing levels of canola meal (Figure 4.3). Additionally, there was no significant difference between the yolk TMA concentrations of eggs from hens of the AA and AT genotypes at any of the levels of canola meal. See Appendix D for individual yolk TMA concentrations.

A significant linear regression was found between yolk TMA concentration and canola meal level for hens of the TT genotype (Table 4.8). The regression equation for the TT genotype is TMA = 0.186CM + 2.613, therefore the calculated CM inclusion level to obtain 4 μg/g yolk TMA is 7%.

Table 4.7 The main effects of genotype, diet, and their interaction on yolk TMA concentration in the second-generation trial.

Main Effect	P Value
Genotype	<0.0001
Diet	0.0019
Interaction	0.0273

Table 4.8 Regression analysis by genotype between yolk TMA concentration and CM inclusion level.

Genotype	P Value	R^2 Value
AA	0.6385	0.0080
TT	0.4414	0.0248
AT	0.0005	0.3599

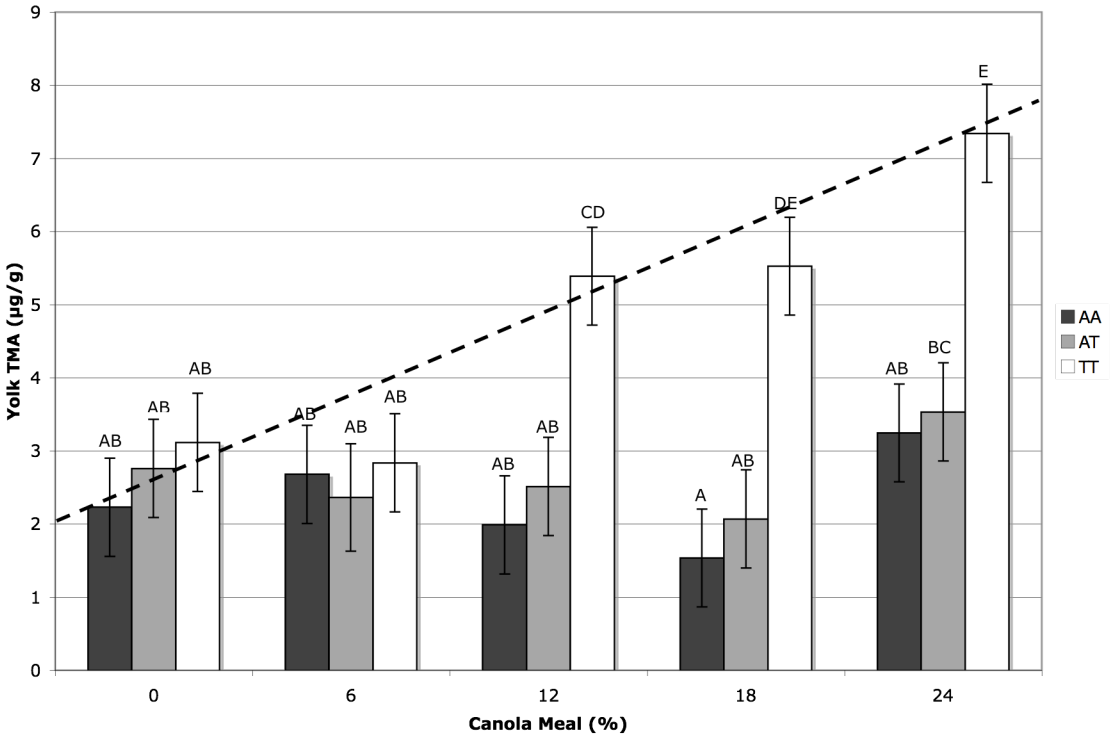

Figure 4.3 The effects of *FMO3 c.984A>T* genotype and canola meal on yolk TMA concentration (µg/g) in the second-generation trial. The dashed line represents the regression equation of the TT genotype. Means with the same letter are not significantly different ($P<0.05$). Error bars indicate the standard error of the mean.

4.2.2.2 Variation

Neither diet, genotype, nor their interaction had significant effects on the CV for yolk TMA concentration (Table 4.9). However, the effect of genotype approached significance, with the AT genotype again demonstrating the highest CV (Table 4.10). See Appendix D for individual CVs.

Table 4.9 The main effects of genotype, diet, and their interaction on the coefficient of variation of yolk TMA concentration in the second-generation trial.

Main Effect	P Value
Genotype	0.0873
Diet	0.3311
Interaction	0.1824

Table 4.10 Coefficient of variation of yolk TMA concentration by genotype in the second-generation trial, where SEM is the standard error of the mean.

Genotype	AA	AT	TT
Coefficient of Variation	41.75	56.26	45.15
SEM	4.71	4.81	4.81

4.2.3 Serum TMA

4.2.3.1 Genotype and Diet

Diet alone did not have a significant effect on serum TMA concentration, however genotype and diet by genotype interaction did (Table 4.11). No clear trend was apparent when the means were separated using the LSD method (Figure 4.4). No significant differences were found within the AA and AT genotypes and only the 0% CM diet was significantly different for the TT genotype. Significant differences within diets were only found for the 12 and 18% CM diets. No significant linear regressions

were found between serum TMA concentration and diet for any of the genotypes (Table 4.12). See Appendix D for individual serum TMA concentrations.

Table 4.11 The main effects of genotype, diet, and their interaction on serum TMA concentration in the second-generation trial.

Main Effect	P Value
Genotype	0.0208
Diet	0.6829
Interaction	0.0044

Table 4.12 Regression, by genotype, between serum TMA concentration and canola meal inclusion level in the second-generation trial.

Genotype	P Value	R^2 Value
AA	0.5024	0.0190
AT	0.6530	0.0080
TT	0.0682	0.1268

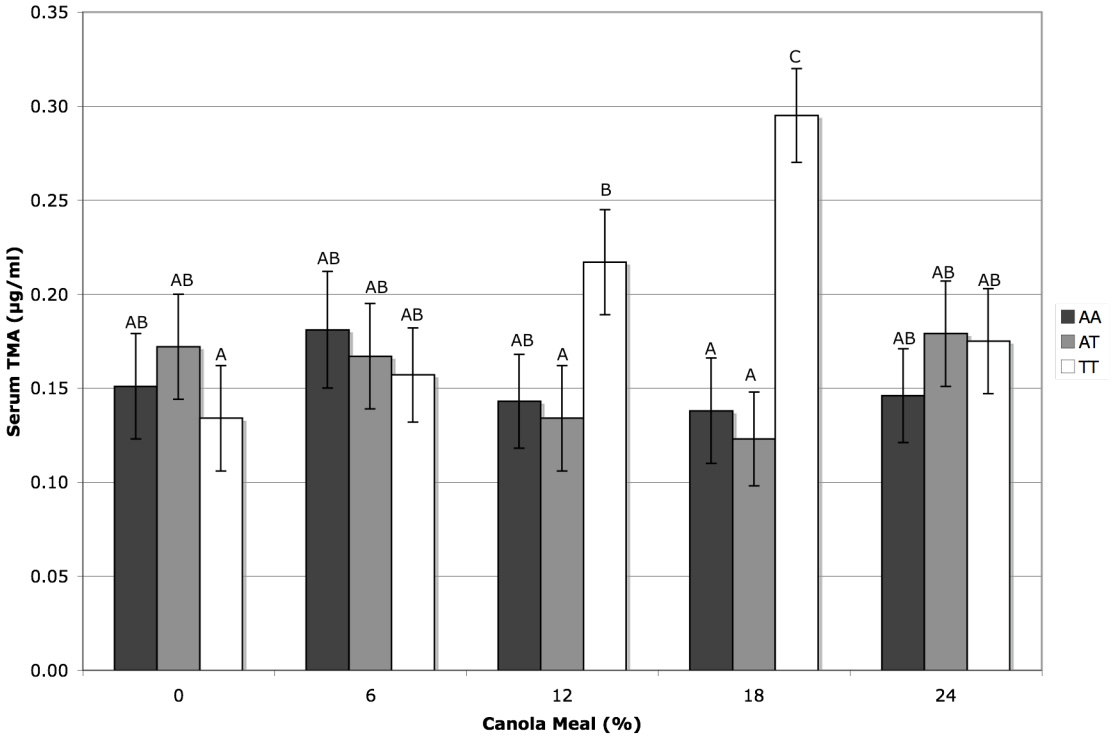

Figure 4.4 The effects of genotype and diet (% canola meal) on serum TMA concentration (µg/ml) in the second-generation trial. Means with the same letter are not significantly different ($P<0.05$). Error bars indicate the standard error of the mean.

4.2.3.2 Correlation With Yolk TMA

No correlation was found between yolk and serum TMA concentration (Table 4.13).

Table 4.13 Correlation between yolk and serum TMA concentrations, both overall and separated by genotype.

Genotype	P value	r Value
AA	0.4840	-0.1436
AT	0.6512	0.0951
TT	0.5889	-0.1088
Overall	0.3817	0.1044

4.2.4 Production Characteristics

Rate of lay and average daily gain (ADG) were analyzed to observe if *FMO3 c.984A>T* genotype affects production characteristics. ADG was calculated as the final body weight minus the initial body weight divided by the number of days on trial. Rate of lay was calculated as the total number of eggs laid divided by the hen days (number of days for which egg production was recorded). Only the total feed consumed per diet was recorded (Table 4.14), therefore statistical analyses could not be performed. Average daily intake (ADI) was calculated as the total feed consumed divided by the total hen days for that treatment. See Appendix E for individual egg production and body weights.

It should be noted that the short duration of this trial (four weeks) does not provide information about the whole production curve, but does provide enough information to determine if a more detailed production study was warranted. Diet and

genotype did not have significant effects on egg production or ADG (Table 4.15), and therefore a full-scale production trial was not performed.

Table 4.14 Total feed consumption and average daily intake (ADI) for each diet in the second-generation trial.

Diet	ADI (g/bird/day)
0% CM	102
6% CM	103
12% CM	105
18% CM	99
24% CM	104
0.055% ChCl	104
0.11% ChCl	98
0.165% ChCl	99
0.22% ChCl	108

Table 4.15 The main effects of genotype, diet, and their interaction on average daily gain (ADG) and egg production in the second-generation trial.

	P Value	
Main Effect	ADG	Egg Production
Genotype	0.3517	0.6027
Diet	0.2525	0.0915
Interaction	0.5688	0.4773

4.3 Second-Generation Trial: Choline Source and Level

The results of the choline chloride and canola meal diets for hens of the TT genotype are presented in this section. The canola meal diets were the same as those presented in section 4.2; though in this section they will be referred to by their total choline concentration (0.158, 0.187, 0.215, and 0.243%). The control diet (0.130%) was used for both choline sources. It was fed to only one group of TT hens, and was therefore treated as a pseudo-replicate.

4.3.1 Yolk TMA

The effects of choline source, level, and their interaction on yolk TMA concentration were all found to be significant (Table 4.16). There appeared to be no response in yolk TMA concentration to increasing levels of choline chloride; in fact only the 0.187 and 0.243% diets were significantly different (Figure 4.5). In contrast, there was a significant linear regression between choline level and yolk TMA concentration for the canola meal diets (Table 4.17). The regression equation for CM is TMA = 39.411(% choline) – 2.513. See Appendix D for individual yolk TMA concentrations.

Table 4.16 The main effects of choline source, level, and their interaction on yolk TMA concentration.

Main Effect	P Value
Source	0.0002
Level	0.0110
Interaction	0.0196

Table 4.17 Regression, by choline source, between yolk TMA concentration and choline inclusion level.

Choline Source	P Value	R^2 Value
Canola Meal	0.0004	0.3608
Choline Chloride	0.7023	0.0053

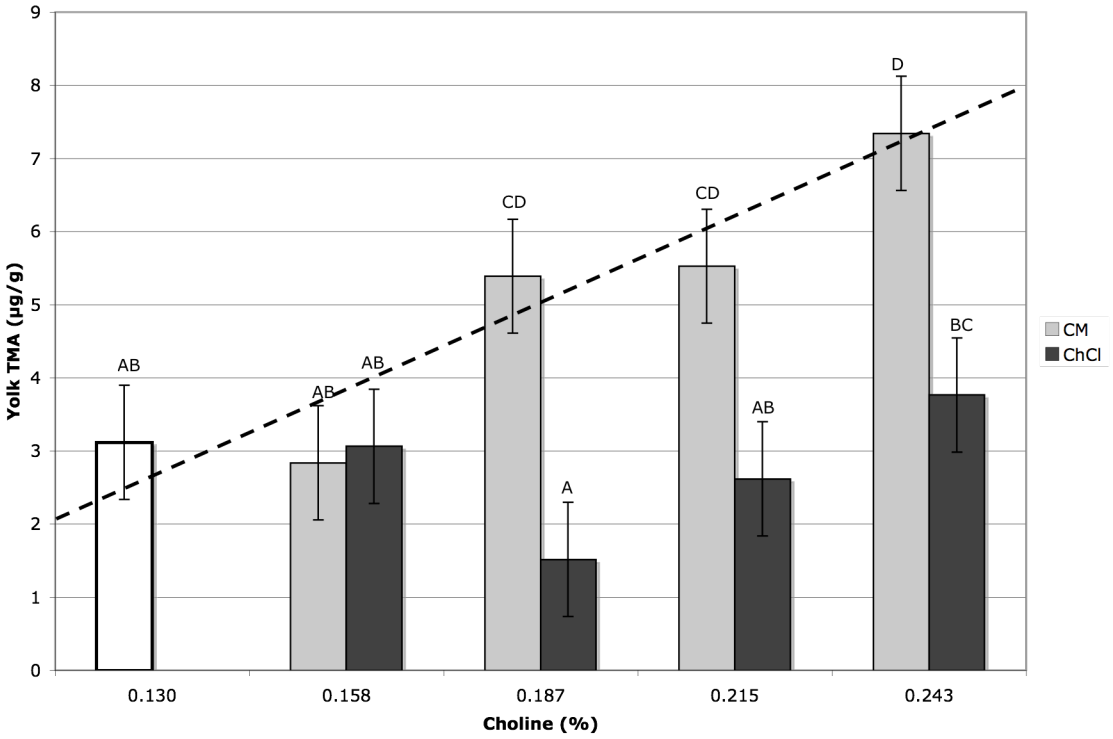

Figure 4.5 The effects of choline source (canola meal or choline chloride) and level on yolk TMA concentration (µg/g) of TT hens in the second-generation trial. The light grey bars represent the canola meal (CM) diets, and the dark grey bars the choline chloride (ChCl) diets. The white bar represents the control diet (0% CM and 0% ChCl), which was pseudo-replicated. The dashed line represents the regression equation for the CM diets. Means with the same letter are not significantly different ($P<0.05$). Error bars indicate the standard error of the mean.

4.4 Other Layer Strains

A summary of the genotypic and allelic frequencies of a one white-shelled laying strain (Lohmann White Leghorn) two commercial brown-shelled (Lohmann Brown and ISA Brown) and is presented in Table 4.18, with individual genotypes presented in Appendix G.

Table 4.18 Genotypic and allelic frequencies of *FMO3 c.984A>T* for two commercial brown-shelled and one commercial white-shelled laying strains, with n as the total number of hens per genotype per strain and WLH as White Leghorn.

	Lohmann Brown		ISA Brown		Lohmann WLH	
Genotype	n	Frequency	n	Frequency	n	Frequency
AA	45	0.45	41	0.44	89	1.00
AT	54	0.54	39	0.42	0	0.00
TT	1	0.01	13	0.14	0	0.00
A		0.72		0.65		1.00
T		0.28		0.35		0.00

5 DISCUSSION

5.1 Allelic and Genotypic Frequencies

The allele frequencies of the first-generation population (Table 4.1) were not in Hardy-Weinberg equilibrium (HWE). This was due to the fact that a three-way cross mating design had been used to produce the first-generation population. Three pure lines are maintained by the breeding company; two of these lines were crossed to produce the hens that were then mated to the third line to produce commercial chicks. The low frequency of the AA genotype (0.01) suggests that the male line was almost fixed for the T allele. The Lohmann hens (Table 4.18) also did not fit HWE. This was expected, as Lohmann International is using selective breeding to eliminate the TT genotype from its commercial strains and therefore mating was not random (Lohmann Tierzucht 2005). Lohmann has been very successful in eliminating the T allele from their commercial brown line, as the frequency of the T allele has decreased from 0.62 (Honkatukia *et al*. 2005) to 0.28 (Table 4.18) in only a few years. In contrast, the allele frequencies of the ISA brown hens (Table 4.18) did fit HWE, indicating that ISA was likely not selecting for *FMO3* in that generation.

5.2 Inheritance of Fishy-Egg Taint

The human detection threshold of TMA in whole-egg homogenate has been estimated to be 1 µg/g (Griffiths *et al.* 1979). Given that TMA is almost exclusively

concentrated in the yolk (Hobson-Frohock *et al*. 1973) and that the yolk makes up approximately 25% of the mass of the total egg contents (Suk and Park 2001), the human detection threshold can be estimated to be 4 μg/g in the yolk. However, there is variability in the ability of individuals to detect TMA (Griffiths *et al*. 1979), and while 4 μg/g is an appropriate benchmark, eggs with yolk TMA concentrations above that are likely to be undetected by the majority of consumers.

Our results showed that when hens were fed diets containing canola meal, including and exceeding those typically found in commercial egg production (Hickling 2001), egg tainting was inherited as a recessive trait. Only hens of the TT genotype displayed a significant increase in yolk TMA with increasing levels of canola meal (Figure 4.3). Also, there was no significant difference between the AA or AT genotypes for any of the diets, confirming that egg tainting is indeed recessive and not additive at these levels of dietary substrate inclusion. These findings are consistent with the recessive inheritance of trimethylaminuria (accumulation of TMA) in humans and dairy cows (Dolphin *et al.* 1997a, Lundén *et al*. 2002b).

Our data did not support our original hypothesis that fishy egg tainting was additive. We hypothesized that the heterozygous hens would be able to metabolize TMA to a limited extent to prevent a detectible odour in the eggs (TMA yolk concentration less than 4 μg/g) but that the yolk TMA concentration would still be higher than that of the homozygous normal hens.

It is likely that the diets used in our trials resulted in low enough TMA production that, in the heterozygous hens, there was sufficient wild-type FMO3 to metabolize the TMA present, but at higher levels of TMA generation the oxidative capabilities of the wild-type FMO3 would become saturated, resulting in excessive

accumulation of TMA in developing follicles. Therefore under commercial production practices (*i.e.* canola meal content less than 24%), TMA generation is sufficiently low enough that egg tainting is recessive.

5.3 Choline Source

Choline is an essential nutrient for laying hens. Deficiency can lead to perosis in chicks (slipped Achilles tendon) and increased liver weight (Budowski *et al.* 1977). Unlike chicks, hens are able to synthesize a large portion of their choline requirements (Crawford *et al.* 1969). The NRC (1994) choline requirements for brown-shelled layers are 0.1225% for chicks, 0.047% for pre-lay pullets, and 0.1050% for laying hens. HyLine (2006) recommends supplementing all grower rations with 0.03% and layer rations with 0.02% additional choline to ensure requirements are met. All of our experimental diets (Table 3.2, Table 3.4) exceeded NRC (1994) choline requirements. Even the second-generation control diet, which was not supplemented with choline chloride, contained 0.130% total choline. This raises the question as to whether laying hen diets really need to be supplemented with choline. Decreasing or even eliminating choline chloride from laying hen diets may allow canola meal to be fed to TT hens at low levels (*i.e.* 5%) without leading to the production of tainted eggs.

Our results are the first to confirm that genotypically predisposed (*FMO3 c. 984* TT) hens do not produce fishy-tainted eggs when fed choline chloride at levels up to 0.220% (Figure 4.5). This confirms the results of Goh *et al*. (1979b), who reported that when taint-producing hens were fed 0.05% choline chloride no tainted eggs were produced, but when the same hens were fed 0.14% sinapine bisulphate (the equivalent of 10% RSM) tainted eggs were produced. Budowski *et al*. (1977) found that when

0.101% choline chloride (in a casein-glucose diet) was fed to 30-day old chicks over 90% was absorbed by the duodenum and upper jejunum. This leaves very little, if any, choline available for fermentation to TMA by bacteria located in the ceca (Emmanuel *et al.* 1984).

As previously stated, Honkatukia *et al.* (2005) and Kretzschmar *et al.* (2007) were able to induce egg tainting in TT hens by feeding high concentrations of choline chloride (0.60% total choline). Choline is transported across the intestinal brush-boarder membrane via facilitated diffusion, a mechanism that can become saturated (Hegazy and Schwenk 1984, Saitoh *et al*. 1992). It is possible that the very high level of inclusion used by Honkatukia *et al.* (2005) and Kretzschmar *et al.* (2007) overwhelmed the absorptive mechanisms in the distal small intestine, allowing more choline chloride to reach, and thus be utilized by, TMA-producing bacteria.

5.4 Serum Versus Yolk Trimethylamine Concentration

Serum TMA concentration was not predictive of yolk TMA concentration. It would have been advantageous if serum and egg TMA concentrations were correlated. This would have enabled both male and female chickens of any age to be evaluated for tainting potential, saving time and resources. The ability to evaluate the tainting potential of males would have been useful in pedigree analysis and selective breeding programs. However, with modern molecular techniques and knowledge of the inheritance pattern under commercial conditions, it is simpler and faster to select against tainting by genotyping for *FMO3 c.984A>T* rather than yolk TMA analysis.

There are several explanations as to why we did not observe a linear correlation between yolk and serum TMA concentrations. Serum TMA concentration only provides

a momentary snap-shot of the TMA profile of an animal, where as yolk TMA shows a longer-term profile. Though the rate of TMA oxidation varied significantly between tainting and non-tainting hens, Pearson *et al*. (1979) found that 24 hours after intravenous administration of ^{14}C-TMA there was no significant difference in ^{14}C-TMA concentration remaining in circulation. Given the short half-life of TMA in circulation, for both tainting and non-tainting hens, time of blood sampling may have had a major impact on our results.

Blood sampling was not performed randomly; we began sampling at approximately 8:30 a.m. and obtained samples from each hen belonging to one treatment before moving on to the next. Sample collection took approximately 5 hours to complete. The 24% CM group was the second to be sampled and the 18%CM group was the last, leading us to believe that time of sampling may be responsible for the unexpectedly low serum TMA concentration found in the 24% CM group. The lights in the barn went on automatically at 4:00 a.m. and the hens always had continuous access to feed, therefore it is unlikely the unexpected serum TMA results were a result of differences in gut fill between the first and last birds sampled. It is still possible, however, that there may be diurnal variations in serum TMA concentration. The rapid phase of yolk development occurs over a period of 6 to 14 days (Marza and Marza 1935); therefore the diurnal variations in circulating TMA levels are unlikely to affect yolk TMA concentrations.

5.5 Day-to-Day Yolk Trimethylamine Variation

Our findings of large day-to-day variations in yolk TMA concentration are supported by previous reports in the literature. Vondell (1932) was the first to note that

tainted eggs were only laid intermittently by some hens, with individual hens displaying large day-to-day variation in the "smelliness" of their eggs. Bolton *et al*. (1976) also noted great variation in egg tainting. They found that only approximately one out of every five eggs laid by tainting hens was tainted.

It was somewhat surprising to find greater individual variation amongst the AT than the TT hens. However, upon further consideration, this pattern can be explained. The CV for each hen was calculated by dividing the standard deviation of the TMA concentration of the three yolks by there mean. If a TT hen and an AT hen, for example, had the same standard deviation but the TT hen had a higher mean the AT hen would have a greater CV.

Another explanation is that the AA hens, with two functional copies of *FMO3*, are fully capable of metabolizing circulating TMA and therefore display the lowest variation of the three genotypes (Table 4.10). The TT hens can only metabolize very little, if any, TMA (Pearson *et al*. 1979, Honkatukia *et al.* 2005). Therefore the primary factor influencing yolk TMA concentration is absorption of TMA from the gut. The AT hens, with only one functional copy of *FMO3*, are still able to metabolize TMA, but to a lesser extent that the AA hens (Kretzschmar *et al.* 2007). It can be assumed that, as such, they are more sensitive to factors that inhibit the oxidative capacity of FMO3, such as glucosinolates. The yolk TMA concentration of AT hens is influenced not only by the amount of TMA absorbed by the gut but also by factors which inhibit FMO3, therefore resulting in greater variation than either AA or TT hens.

The cause of the variation in yolk TMA concentration is still unclear. Marza and Marza (1935) reported day-to-day fluctuations in the rate of yolk growth during the rapid phase of yolk development. It is possible that such variations in yolk growth may

impact TMA accretion in the yolk, resulting in inter-yolk variation in TMA concentration.

There are some differences between the results of the first and second-generation trials. While both trials display the same trends, the yolk TMA concentrations are generally lower in the second-generation trial for the corresponding genotype and canola meal level (Figure 4.2, Figure 4.3). This may be due in part to differences in diet formulation, gut microbiota, and other unknown factors that influence yolk TMA variability.

6 CONCLUSIONS

The purpose of this study was to determine the mode of inheritance of fishy-egg tainting when brown-shelled hens are fed diets containing canola meal at commercial levels of inclusion. We have definitively shown that egg tainting is recessively inherited when hens are fed canola meal up to levels of 24%. There is no "safe" level of canola meal that can be fed to affected hens without the risk of egg tainting; however, low levels (such as 6%) may be used if the producer is willing to accept the risk that a small portion of the eggs will be tainted. Also, we have found that choline chloride, at and slightly above levels typical to commercial production, does not cause fishy-egg tainting.

With the availability of molecular testing, and the inheritance pattern now clear, breeding companies will be able to remove tainting from their flocks. The recessive nature of the trait is advantageous to primary breeding companies, as they will only need to select against the T allele in one, rather than both, of their parental lines. If the parental line to be selected against is a production of two pure grandparent lines (which is likely to be the case as commercial laying hen lines are often the product of a three or four-way cross) then the T allele must be removed from both grandparental lines.

The elimination of tainting hens from commercial lines will allow brown-shelled egg producers to feed canola meal, an economical feed ingredient, without worry of producing tainted eggs. This will expand export markets for Canadian canola meal to brown-shelled egg producers around the world.

Large day-to-day variations in yolk TMA concentration are present in hens of all three genotypes. The cause of this variation is still unclear, and further research is required. It may be of interest to examine changes in the cecal microbiota over time and perhaps factors that may influence choline absorption or yolk deposition. As well, infusions of C^{14} labeled TMA could be utilized to examine how TMA accumulates

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APPENDIX A

First-generation Genotypes

Lab #	Wing Band#	Genotype
201	1840	AT
202	1810	AT
203	1871	AT
204	1895	AT
205	1811	AT
206	1834	AT
207	1861	TT
208	1866	AT
209	1862	AT
210	1880	AT
211	1804	AT
212	1872	AT
213	1805	-
214	1806	AT
215	1817	AT
216	1863	AT
217	1858	AT
218	1898	AT
219	1855	TT
220	1842	AT
221	1854	AT
222	1815	AT
223	1801	AT
224	1886	TT
225	1888	AT
226	1802	AT
227	1900	AT
228	1850	AT
229	1856	AT
230	1843	TT
231	1813	TT
232	1897	AT
233	1833	TT
234	1853	TT
235	1808	AT

Lab #	Wing Band#	Genotype
236	1891	AT
237	1829	AT
238	1894	AT
239	1859	AT
240	1846	AT
241	1803	TT
242	1848	AT
243	1844	AT
244	1851	TT
245	1812	AT
246	1825	AT
247	1896	AT
248	1826	AT
249	1889	TT
250	1870	TT
251	1836	AT
252	1824	AT
253	1835	AT
254	1892	AT
255	1869	AT
256	1830	AT
257	1832	AT
258	1822	AT
259	1881	AT
260	1841	AT
261	1831	AT
262	1899	TT
263	1847	AT
264	1816	AT
265	1867	AT
266	1883	TT
267	1818	AT
268	1885	AT
269	1887	AT
270	1882	AT

Lab #	Wing Band#	Genotype
275	1857	AT
276	1868	AT
277	1860	AT
278	1865	AT
279	1873	AT
280	1814	AT
281	1875	AT
282	1807	TT
283	1879	TT
284	1839	AT
285	1852	AT
286	1884	AT
287	1877	AT
288	1823	AT
289	1864	AT
290	1849	TT
291	1827	AT
292	1893	AT
293	1837	AT
294	1819	TT
295	1820	AT
296	1828	AT
297	1838	AT
298	1809	AT
299	1878	AT
300	1845	AT
301	1993	AT
302	1938	AT
303	1944	TT
304	1942	AT
305	1965	AT
306	1989	AT
307	1922	AT
308	1975	AT
309	1925	AT
310	1934	AA
311	1914	AT
312	1991	AT
313	1941	AT
314	1970	AT
315	1937	AT
316	1960	AT
317	1988	AT

Lab #	Wing Band#	Genotype
318	1931	AT
319	1978	TT
320	1982	AT
321	1963	TT
322	1967	AT
323	1952	TT
324	1916	AA
325	1901	AT
326	1940	AT
327	1924	AT
328	1905	AT
329	1902	AT
330	1987	AT
331	1953	AT
332	1939	AT
333	1992	AT
334	1980	AT
335	1933	AA
336	1936	TT
337	1964	AT
338	1903	TT
339	1981	AT
340	1935	TT
341	1956	TT
342	1979	AT
343	1918	AT
344	1910	TT
345	1906	AT
346	1998	AT
347	1986	AT
348	1943	AT
349	1974	TT
350	1976	AT
351	1962	AT
352	1904	AT
353	1917	AT
354	1977	AT
355	1973	AA
356	1949	TT
357	1945	TT
358	1929	TT
359	1969	TT
360	2000	AT

Lab #	Wing Band#	Genotype
361	1999	AT
362	1928	AT
363	1911	AT
364	1913	AT
365	1966	AT
366	1959	TT
367	1983	AT
368	1950	TT
369	1951	TT
370	1915	AT
371	1907	TT
372	1955	AT
373	1994	AT
374	1919	TT
375	1971	AT
376	1957	TT
377	1958	TT
378	1995	AT
379	1923	AT
380	1908	TT
381	1912	AT
382	1909	AT
383	1930	AT
384	1946	AT
385	1948	AT
386	1972	AT
387	1984	AT
388	1954	AT
389	1996	AT
390	1921	AT
391	1927	AT
392	1947	AT
393	1932	AT
394	1920	AT
395	1926	AT
396	1961	AT
397	1997	AT
398	1968	AT
399	1990	AT
400	1985	AT
401	2100	TT
402	2091	AT
403	2034	AT

Lab #	Wing Band#	Genotype
404	2044	TT
405	2086	TT
406	2048	AT
407	2017	TT
408	2005	AT
409	2001	AT
410	2003	TT
411	2079	AT
412	2019	AT
413	2057	AT
414	2008	AT
415	2033	AT
416	2070	TT
417	2027	AA
418	2037	AT
419	2072	TT
420	2084	AT
421	2023	TT
422	2028	AT
423	2083	AT
424	2095	AT
425	2036	AT
426	2075	AT
427	2025	AT
428	2006	AT
429	2094	AT
430	2050	AT
431	2042	TT
432	2043	AT
433	2002	AT
434	2026	AT
435	2069	AT
436	2047	AT
437	2004	AT
438	2093	AT
439	2011	AT
440	2039	AT
441	2021	TT
442	2022	AT
443	2085	AT
444	2012	AT
445	2078	AT
446	2010	AT

Lab #	Wing Band#	Genotype
447	2015	TT
448	2013	AT
449	2089	AT
450	2096	TT
451	2088	AT
452	2007	AT
453	2018	AT
454	2067	TT
455	2080	AT
456	2092	AT
457	2061	AT
458	2031	AT
459	2014	TT
460	2076	AT
461	2045	AT
462	2040	AT
463	2030	AT
464	2060	TT
465	2087	AT
466	2090	TT
467	2081	AT
468	2038	AT
469	2029	AT
470	2058	AT
471	2032	AT
472	2066	TT
473	2009	AT
474	2056	AT
475	2064	AT
476	2082	AT
477	2016	AT
478	2098	AT
479	2051	AT
480	2097	AT
481	2035	AT
482	2049	AT
483	2063	AT
484	2068	AT
485	2065	AT
486	2054	AT
487	2024	AT
488	2071	TT
489	2099	TT

Lab #	Wing Band#	Genotype
490	2059	AT
491	2041	AT
492	2073	AT
493	2062	AT
494	2074	TT
495	2046	AT
496	2020	AT
497	2055	TT
498	2077	AT
499	2052	AT
500	2053	TT
501	2186	AT
502	2156	AA
503	2178	TT
504	2162	TT
505	2164	TT
506	2190	AT
507	2115	AT
508	2143	AT
509	2102	AT
510	2145	AT
511	2153	AT
512	2188	AT
513	2169	AT
514	2187	AT
515	2127	TT
516	2147	AT
517	2146	AT
518	2130	AT
519	2113	AT
520	2131	AT
521	2166	AT
522	2120	TT
523	2149	AT
524	2109	AT
525	2175	AT
526	2180	AT
527	2106	AT
528	2139	AT
529	2150	TT
530	2198	AT
531	2110	AT
532	2121	AT

Lab #	Wing Band#	Genotype
533	2129	TT
534	2116	TT
535	2195	AT
536	2107	AT
537	2189	AT
538	2201	AT
539	2158	AT
540	2117	AT
541	2192	TT
542	2124	AT
543	2108	AT
544	2191	AT
545	2103	AT
546	2171	AT
547	2135	AT
548	2122	TT
549	2128	AT
550	2105	AT
551	2161	AT
552	2155	TT
553	2183	AT
554	2119	AT
555	2181	TT
556	2144	AT
557	2151	AT
558	2185	AT
559	2114	AT
560	2174	TT
561	2138	AT
562	2141	AT
563	2196	AT
564	2176	TT
565	2152	AT
566	2199	AT
567	2172	AT
568	2140	AT
569	2136	AT
570	2133	AT
571	2142	TT
572	2173	AT
573	2194	AT
574	2154	AT
575	2125	AT

Lab #	Wing Band#	Genotype
576	2118	AT
577	2126	AT
578	2184	AT
579	2177	TT
580	2182	AT
581	2167	AA
582	2104	AT
583	2179	AT
584	2148	AT
585	2165	AT
586	2163	AT
587	2132	AT
588	2160	TT
589	2134	AT
590	2111	TT
591	2159	AT
592	2168	AT
593	2101	AT
594	2123	AT
595	2137	AT
596	2193	AT
597	2197	AT
598	2157	AT
599	2200	AT
600	2112	AT
601	2267	AT
602	2203	AT
603	2211	AT
604	2292	AT
605	2216	AT
606	2245	AT
607	2273	AT
608	2208	AT
609	2232	AT
610	2253	TT
611	2244	TT
612	2230	AT
613	2254	AT
614	2231	AT
615	2288	AT
616	2281	AT
617	2215	AT
618	2261	AT

Lab #	Wing Band#	Genotype
619	2243	TT
620	2252	AT
621	2294	AT
622	2285	AT
623	2299	AT
624	2260	TT
625	2227	AT
626	2293	TT
627	2263	AT
628	2298	AT
629	2246	AT
630	2204	AT
631	2218	TT
632	2269	AT
633	2284	AT
634	2287	AT
635	2297	AT
636	2226	AT
637	2257	TT
638	2240	TT
639	2251	AT
640	2278	AT
641	2258	-
642	2279	-
643	2250	AT
644	2266	AT
645	2233	AT
646	2289	AT
647	2209	AT
648	2248	AT
649	2241	AT
650	2207	TT
651	2280	AT
652	2259	AT
653	2291	AT
654	2300	AT
655	2236	TT
656	2223	AT
657	2270	TT
658	2301	AT
659	2272	AT
660	2214	AT
661	2283	AT

Lab #	Wing Band#	Genotype
662	2282	AT
663	2275	AT
664	2222	AT
665	2276	AT
666	2286	AT
667	2217	AT
668	2235	AT
669	2213	TT
670	2271	AT
671	2238	AT
672	2247	AT
673	2205	AT
674	2265	AT
675	2264	AT
676	2238	TT
677	2221	AT
678	2249	AT
679	2268	AT
680	2239	AT
681	2234	AT
682	2296	TT
683	2229	AT
684	2274	TT
685	2237	AT
686	2262	AT
687	2255	AT
688	2295	AT
689	2202	AT
690	2220	AT
691	2206	TT
692	2219	AT
693	2277	AT
694	2224	AT
695	2256	AT
696	2212	AT
697	2225	AT
698	2210	AT
699	2290	AT
700	2242	AT
701	2326	AT
702	2370	TT
703	2324	TT
704	2307	TT

Lab #	Wing Band#	Genotype
705	2305	AT
706	2361	AT
707	2360	AT
708	2386	AT
709	2381	TT
710	2317	AT
711	2364	AT
712	2333	AT
713	2392	AT
714	2344	TT
715	2385	TT
716	2334	AT
717	2390	AT
718	2349	AT
719	2319	AT
720	2337	AT
721	2387	AT
722	2369	AT
723	2314	TT
724	2394	AT
725	2325	AT
726	2357	AT
727	2353	AT
728	2309	TT
729	2303	AT
730	2329	AT
731	2336	TT
732	2311	TT
733	2376	AT
734	2375	TT
735	2366	AT
736	2350	AT
737	2341	TT
738	2373	AT
739	2356	AT
740	2358	AT
741	2371	AT
742	2331	TT
743	2302	AT
744	2312	TT
745	2354	AT
746	2323	AT
747	2374	AT

Lab #	Wing Band#	Genotype
748	2372	AT
749	2332	AT
750	2313	AT
751	2321	AT
752	2379	AT
753	2359	AT
754	2377	TT
755	2330	AT
756	2382	AT
757	2345	AT
758	1744	AT
759	2322	AT
760	2362	AT
761	2389	AT
762	2338	AT
763	2384	AT
764	2368	TT
765	2306	TT
766	2400	TT
767	2388	AT
768	2380	AT
769	2343	AT
770	2399	AT
771	2383	AT
772	2391	AT
773	2335	TT
774	2315	AT
775	2320	AT
776	2328	AT
777	2395	AT
778	2348	AT
779	2352	AT
780	2396	AT
781	2367	AT
782	2365	AT
783	2363	AT
784	2308	AT
785	2346	AT
786	2351	AT
787	2347	AT
788	2355	AT
789	2327	AT
790	2339	TT

Lab #	Wing Band#	Genotype
791	2316	AT
792	2340	TT
793	2310	AT
794	2398	AT
795	1743	TT

Lab #	Wing Band#	Genotype
796	2304	TT
797	2342	AT
798	2318	AT
799	2393	AT
800	2378	AT

APPENDIX B

First-generation Trial Trimethylamine Data

Cage #	Wing Band #	Geno-type	Diet (% CM)	Yolk TMA (µg/g)			CV
				Egg A	Egg B	Egg C	
871	2395	AT	18	64.99	35.96	55.70	27.71
872	2010	AT	18	61.97	23.05	192.00	91.86
873	1990	AT	18	46.57	19.43	52.35	43.93
875	2389	AT	18	40.52	19.03	65.60	59.40
877	2376	AT	18	.	.	.	.
1101	1801	AT	0	17.92	40.72	95.60	83.75
1102	1840	AT	0	21.99	31.86	44.51	33.10
1103	1810	AT	0	58.30	21.83	175.15	92.24
1104	1853	AT	0	56.45	48.59	58.85	13.09
1105	1889	TT	0	43.53	55.70	45.12	4.31
1106	1880	AT	0	76.94	50.74	29.04	51.28
1107	1939	AT	0	46.71	22.63	54.12	43.27
1108	1833	TT	0	79.02	19.13	82.34	60.83
1109	1862	AT	0	63.87	34.71	50.75	30.37
1110	1931	AT	0	77.90	125.76	60.74	33.82
1111	2243	TT	0	22.61	23.68	53.38	46.77
1112	2218	TT	0	43.63	54.28	42.52	12.89
1113	2244	TT	0	22.08	87.12	56.99	56.51
1114	2331	TT	0	18.06	8.55	55.69	67.52
1115	2307	TT	0	22.07	48.98	63.63	43.16
1116	2385	TT	0	16.33	24.56	47.99	59.80
1117	2270	TT	0	16.35	17.11	84.43	93.27
1118	2368	TT	0	19.82	19.71	56.59	59.77
1119	2346	AT	0	32.17	79.30	46.90	41.16
1121	1831	AT	6	.	.	.	.
1122	1879	TT	6	64.21	51.32	61.39	9.12
1123	1883	TT	6	79.57	87.64	95.69	12.36
1124	1816	AT	6	16.35	25.11	59.84	60.86
1125	1881	AT	6	19.39	24.94	58.45	63.79
1126	1841	AT	6	.	.	.	.
1127	1878	AT	6	19.55	41.41	46.69	40.75
1128	1954	AT	6	33.35	27.07	36.76	38.81
1129	1830	AT	6	17.97	36.22	49.79	49.01
1130	1868	AT	6	20.29	60.88	52.92	47.16
1131	2176	TT	6	50.31	37.77	65.07	24.10

Cage #	Wing Band #	Geno-type	Diet (% CM)	Yolk TMA (µg/g)			CV
				Egg A	Egg B	Egg C	
1132	2181	TT	6	38.39	19.32	54.06	27.69
1133	2177	TT	6	27.70	25.35	56.88	49.78
1134	1819	TT	6	95.55	36.39	87.21	43.04
1135	2160	TT	6	103.10	.	.	.
1136	2014	TT	6	69.24	50.31	96.19	29.05
1137	2024	AT	6	49.55	44.28	65.99	19.82
1138	1929	TT	6	106.39	57.64	88.91	30.28
1139	1951	TT	6	162.31	123.52	155.19	15.83
1140	2053	TT	6	60.63	51.06	88.12	27.06
1141	2100	TT	12	49.19	88.80	119.79	37.67
1142	2003	TT	12	63.53	77.93	105.08	26.98
1143	2072	TT	12	135.93	77.05	124.15	29.77
1144	1963	TT	12	139.38	117.96	130.73	12.71
1145	1952	TT	12	116.39	137.40	251.79	40.63
1146	2218	TT	12	25.18	24.85	54.36	47.80
1147	1978	TT	12	81.00	118.70	153.56	32.79
1148	2293	TT	12	29.54	49.91	87.50	71.50
1149	1969	TT	12	107.03	128.97	119.81	2.80
1150	1958	TT	12	47.24	75.38	117.82	41.04
1151	1925	AT	12	20.73	18.72	60.32	72.17
1152	1975	AT	12	15.69	13.74	54.15	78.80
1153	1953	AT	12	19.31	18.31	56.15	68.49
1154	1940	AT	12	19.94	30.90	74.41	61.66
1155	1814	AT	12	21.56	51.54	65.02	43.51
1156	1845	AT	12	21.44	52.47	26.44	53.65
1157	1822	AT	12	19.34	63.67	63.73	52.05
1158	1867	AT	12	25.94	29.18	77.05	66.92
1159	1818	AT	12	16.34	24.49	26.23	23.06
1160	1837	AT	12	20.93	79.97	56.17	55.82
1161	2238	TT	18	178.90	220.42	210.84	19.72
1162	2377	TT	18	159.25	121.58	116.02	23.53
1163	2304	TT	18	89.29	124.35	111.32	18.31
1164	2400	TT	18	43.55	63.90	71.15	28.02
1165	2174	TT	18	85.33	142.18	110.56	21.53
1166	2142	TT	18	183.41	264.20	279.85	18.14
1167	2178	TT	18	157.11	123.77	129.20	16.60
1168	2164	TT	18	127.82	84.02	121.17	24.67
1169	2074	TT	18	98.84	113.38	157.00	29.55
1170	2055	TT	18	121.59	132.49	142.77	53.85
1171	1928	AT	18	21.08	65.52	58.90	50.74
1172	1913	AT	18	16.92	15.56	55.12	80.45
1173	1947	AT	18	23.50	22.80	27.20	4.44
1174	1961	AT	18	27.84	28.73	25.51	0.00

Cage #	Wing Band #	Geno-type	Diet (% CM)	Yolk TMA (µg/g)			CV
				Egg A	Egg B	Egg C	
1175	1995	AT	18	25.22	66.23	64.75	46.30
1176	1946	AT	18	21.28	39.84	68.58	54.09
1177	2000	AT	18	130.44	25.86	59.29	69.99
1178	1999	AT	18	17.39	24.05	60.88	62.84
1179	1989	AT	18	23.34	26.21	30.25	4.02
1180	1902	AT	18	101.83	132.41	106.56	17.55

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APPENDIX C

Second-generation Genotypes

Lab #	Wing Band #	Genotype
101	1805	AA
102	1806	AA
103	1801	AT
104	1803	TT
105	1802	AT
106	1804	AT
107	1809	AA
108	1807	AT
109	1808	AT
110	1819	AT
111	1813	TT
112	1817	TT
113	1810	TT
114	1811	AA
115	1814	AT
116	1816	AT
117	1815	AT
118	1818	AA
119	1812	AT
120	1821	AT
121	1826	TT
122	1822	TT
123	1824	AT
124	1823	AT
125	1820	TT
126	1825	AT
127	1835	AA
128	1841	TT
129	1832	AA
130	1837	AT
131	1838	AT
132	1829	AA
133	1828	AT
134	1830	AT

Lab #	Wing Band #	Genotype
135	1839	TT
136	1831	AT
137	1834	AT
138	1840	AA
139	1827	AT
140	1833	TT
141	1836	AA
142	1843	AT
143	1842	
144	1844	TT
145	1845	AT
146	1851	AT
147	1850	TT
148	1848	TT
149	1847	AA
150	1849	TT
151	8058	AT
152	1846	AT
153	1853	TT
154	1855	AT
155	1860	AA
156	1862	AT
157	1856	AA
158	1863	TT
159	1857	AA
160	1861	TT
161	1859	AT
162	1858	AT
163	1864	AA
164	1854	AT
165	1865	AA
166	1870	AA
167	1869	AT
168	1866	AT

Lab #	Wing Band #	Genotype
169	1867	AT
170	1868	AA
171	1881	AT
172	1880	AT
173	1871	AA
174	1877	TT
175	1879	AT
176	1873	AT
177	1884	AT
178	1875	AT
179	1882	TT
180	1885	AT
181	1872	TT
182	1883	AA
183	1878	AT
184	1876	AT
185	1874	AT
186	1891	AA
187	1894	AA
188	1895	AT
189	1889	AT
190	1893	AT
191	1897	AT
192	1896	AT
193	1888	AT
194	1899	AT
195	1890	AA
196	1892	AA
197	1887	AA
198	1900	AT
199	1898	AT
200	1886	AA
201	1912	AT
202	1902	AT
203	1911	AT
204	1901	AT
205	1906	TT
206	1904	AT
207	1909	TT
208	1905	AT
209	1910	AT
210	5352	AT
211	1908	TT

Lab #	Wing Band #	Genotype
212	1913	-
213	1907	AT
214	1921	-
215	1918	AT
216	1926	AT
217	1925	AA
218	1924	AT
219	1916	AT
220	1919	AT
221	1922	AT
222	1920	AT
223	1927	AT
224	1923	AA
225	1917	TT
226	1915	AT
227	1939	TT
228	1940	TT
229	1932	TT
230	1938	TT
231	1933	TT
232	1936	TT
233	1934	TT
234	1930	TT
235	1929	TT
236	1942	TT
237	1941	AT
238	1928	TT
239	1935	TT
240	1937	TT
241	1931	TT
242	1950	TT
243	1945	TT
244	1954	AT
245	1949	AT
246	1951	TT
247	1943	AT
248	1946	AT
249	1948	AT
250	1944	TT
251	1955	AT
252	1947	TT
253	1953	TT
254	1952	TT

Lab #	Wing Band #	Genotype
255	1970	AT
256	1961	TT
257	1962	TT
258	1965	TT
259	1959	AT
260	5350	AT
261	1963	AT
262	1956	TT
263	1967	TT
264	1966	TT
265	1972	TT
266	1958	AT
267	1971	TT
268	1964	TT
269	1968	TT
270	1957	AT
271	1969	AT
272	1979	TT
273	1982	AT
274	1977	TT
275	1978	AT
276	1984	TT
277	1980	AT
278	1975	TT
279	1981	TT
280	1973	AT
281	1976	TT
282	1985	TT
283	1983	TT
284	1974	AT
285	1987	TT
286	1990	AT
287	1989	AT
288	1993	TT
289	1991	TT
290	1998	AA
291	5349	AT
292	1999	TT
293	1986	TT
294	1996	TT
295	1992	TT
296	1997	TT
297	1994	TT

Lab #	Wing Band #	Genotype
298	1995	AT
299	2000	TT
300	2004	TT
301	2003	AT
302	2005	AT
303	2002	TT
304	2001	TT
305	2006	AT
306	2010	TT
307	2008	AT
308	2007	TT
309	2009	AT
310	2020	AT
311	2012	AA
312	2022	AA
313	2017	TT
314	2021	AA
315	2011	AA
316	2013	TT
317	2025	TT
318	2014	AT
319	2024	TT
320	2019	AT
321	2018	AT
322	2016	TT
323	2015	AT
324	2023	AT
325	2028	AT
326	2030	AT
327	2026	TT
328	2027	AT
329	2034	AT
330	2031	AT
331	2035	AT
332	2033	TT
333	2029	AT
334	2032	TT
335	2044	TT
336	2043	AT
337	2047	AT
338	2038	AT
339	2039	AA
340	2041	AT

Lab #	Wing Band #	Genotype
341	2037	AT
342	2045	TT
343	2036	AT
344	2048	AT
345	2040	TT
346	2042	AT
347	2046	AT
348	2071	AA
349	2063	AT
350	2067	AT
351	2073	AA
352	2070	AT
353	2064	AT
354	2072	AT
355	2062	AT
356	2065	AT
357	2068	TT
358	2066	TT
359	2076	AT
360	2077	AA
361	2079	TT
362	2080	AA
363	2078	AA
364	2074	TT
365	2075	AA
366	2090	AA
367	2087	AA
368	2082	AT
369	2085	TT
370	2081	AT
371	2094	AA
372	2091	TT
373	2089	TT
374	2084	AA
375	2086	AA
376	2083	AA
377	2088	AA
378	2093	AT
379	2092	AT
380	2095	TT
381	2104	AT
382	2097	TT
383	2109	AA

Lab #	Wing Band #	Genotype
384	2098	AA
385	2105	AT
386	2107	AT
387	2096	TT
388	2106	AT
389	2101	AA
390	2100	AA
391	2102	TT
392	2108	AT
393	2103	AT
394	2099	AA
395	2111	AA
396	2118	TT
397	2121	AT
398	2122	AT
399	2112	TT
400	2117	AT
401	2116	AT
402	2123	AT
403	2115	AA
404	2114	TT
405	2120	AT
406	2125	TT
407	2119	AA
408	2124	AA
409	2110	AT
410	2113	AT
411	2138	AT
412	2142	AT
413	2141	AA
414	2140	TT
415	2139	AA
416	2157	AA
417	2158	TT
418	2160	AT
419	2159	AT
420	2156	AT
421	2162	AT
422	2155	AA
423	2161	AT
424	2164	AT
425	2163	AT
426	2147	AT

Lab #	Wing Band #	Genotype
427	2154	AT
428	2148	TT
429	2152	AT
430	2151	AT
431	2146	AA
432	2149	AT
433	2143	AA
434	2153	TT
435	2145	AA
436	2150	AT
437	2144	AT
438	2183	AT
439	2185	AT
440	2186	TT
441	2188	AT
442	2184	AT
443	2190	TT
444	2189	AT
445	2192	AT
446	2187	TT
447	2191	AT
448	2200	AT
449	2201	AA
450	2202	AA
451	2204	AA
452	2196	AA
453	2195	TT
454	2203	TT
455	2197	AT
456	2198	AA
457	2199	AT
458	2194	TT
459	2193	TT
460	2218	AA
461	2208	TT
462	2207	AT
463	2215	AT
464	2205	AT
465	2206	AA
466	2217	AT
467	2209	AT
468	2213	AA
469	2214	TT

Lab #	Wing Band #	Genotype
470	2212	AT
471	2216	TT
472	2211	AT
473	2239	AT
474	2233	AT
475	2232	AT
476	2241	AA
477	2237	AT
478	2231	TT
479	2235	AT
480	2238	AT
481	2240	TT
482	2234	AT
483	2236	AT
484	2248	AT
485	2242	AA
486	2253	AT
487	2250	AA
488	2246	TT
489	2252	AT
490	2247	AT
491	2245	AT
492	2243	AT
493	2249	TT
494	2254	AA
495	2251	AT
496	2244	AA
497	2273	AA
498	2271	AA
499	2266	TT
500	2267	AA
501	2268	AA
502	2269	AT
503	2276	AT
504	2278	AA
505	2275	TT
506	2274	AT
507	2279	AA
508	2272	AT
509	2270	AA
510	2277	AT
511	2265	AT
512	2295	AT

Lab #	Wing Band #	Genotype
513	2291	AA
514	2293	AT
515	2297	AT
516	2296	AT
517	2298	AT
518	2301	AT
519	2300	AA
520	2299	AA
521	2294	AT
522	2292	AT
523	2307	AT
524	2309	AA
525	2306	AA
526	2311	AT
527	2304	AT
528	2318	AT
529	2315	AA
530	2313	AT
531	2308	AT
532	2302	TT
533	2316	AA
534	2305	TT
535	2312	AT
536	2317	AT
537	2310	TT
538	2303	TT
539	2314	TT
540	2343	TT
541	2338	TT
542	2347	AT
543	2344	AT
544	2342	AT
545	2334	AT
546	2348	AT
547	2337	AT
548	2341	TT
549	2335	AT
550	2340	AA
551	2339	AA
552	2345	AT
553	2336	AT
554	2346	AT
555	2350	AT

Lab #	Wing Band #	Genotype
556	2356	TT
557	2349	TT
558	2357	AT
559	2352	AA
560	2355	AT
561	2353	AA
562	2351	TT
563	2354	AT
564	2379	TT
565	2382	TT
566	2378	AA
567	2381	AT
568	2380	AA
569	2397	AA
570	2388	AT
571	2396	AT
572	2387	AA
573	2383	AA
574	2394	AA
575	2385	AA
576	2386	TT
577	2389	TT
578	2391	AA
579	2392	AA
580	2395	TT
581	2390	AT
582	2393	AA
583	2398	AT
584	2384	AT
585	2400	AA
586	2404	TT
587	2406	AT
588	2409	AT
589	2414	AT
590	2412	TT
591	2411	TT
592	2403	AT
593	2399	AA
594	2413	AT
595	2408	TT
596	2402	AT
597	2405	AA
598	2401	TT

Lab #	Wing Band #	Genotype
599	2407	TT
600	2410	AT
601	2442	AA
602	2449	AA
603	2441	AT
604	2439	AA
605	2446	TT
606	2440	TT
607	2448	AA
608	2447	TT
609	2445	AT
610	2443	TT
611	2444	TT
612	2474	TT
613	2467	AT
614	2465	TT
615	2464	AA
616	2463	AA
617	2470	AT
618	2473	AA
619	2468	AA
620	2469	AT
621	2471	TT
622	2472	TT
623	2466	AT
624	2462	TT
625	2500	TT
626	2496	AA
627	2499	AA
628	2497	AA
629	2491	TT
630	2493	AT
631	2494	TT
632	2489	AT
633	2495	AT
634	2492	AT
635	2490	TT
636	2501	AT
637	2506	TT
638	2507	TT
639	2502	AT
640	2505	TT
641	2503	AT

Lab #	Wing Band #	Genotype
642	8059	AT
643	2482	AT
644	2477	AA
645	2481	TT
646	2475	AT
647	2480	AA
648	2476	AT
649	2484	AT
650	2525	TT
651	2524	AT
652	2532	TT
653	2527	AA
654	2531	TT
655	2530	AT
656	2529	TT
657	2528	TT
658	2526	TT
659	2546	AT
660	2549	AT
661	2548	AT
662	2552	AT
663	2550	AT
664	2551	AT
665	2547	AA
666	2557	AT
667	2555	AT
668	2559	AT
669	2554	AT
670	2558	TT
671	2556	AA
672	2553	AT
673	2561	AT
674	2564	AT
675	2565	AT
676	2562	AT
677	2560	AT
678	2563	AT
679	2566	AA
680	2576	AT
681	2579	AA
682	2574	AA
683	2578	AT
684	2577	AT

Lab #	Wing Band #	Genotype
685	2575	AT
686	2580	TT
687	2594	AT
688	2593	AA
689	2588	AT
690	2590	AT
691	2589	AT
692	2591	AT
693	2592	AT
694	2597	AT
695	2596	AT
696	2595	AT
697	2598	TT
698	2601	TT
699	2600	TT
700	2599	AA
701	2615	AA
702	2612	AA
703	2614	TT
704	2610	TT
705	2609	AT
706	2613	AT
707	2611	AT
1203	2616	AA
1204	2618	AT
1205	2617	AT
1206	2619	AT
1207	2620	TT
1208	2621	TT
1209	2622	AA
1210	2602	AT
1211	2604	AA
1212	2603	TT
1213	2606	TT
1214	2607	AT
1215	2605	TT
1216	2608	AT
1217	2587	AT
1218	2585	AT
1219	2586	AT
1220	2581	AA
1221	2582	AT
1222	2584	TT

Lab #	Wing Band #	Genotype
1223	2583	AT
1224	2572	AT
1225	2569	TT
1226	2568	AT
1227	2571	AT
1228	2573	AT
1229	2567	AA
1230	2570	TT
1231	2543	AA
1232	2540	AA
1233	2545	AT
1234	2544	TT
1235	2542	AT
1236	2539	AT
1237	2541	AA
1238	2516	AA
1239	2515	AT
1240	2517	AT
1241	2519	AT
1242	2514	AT
1243	2518	TT
1244	2513	TT
1245	2522	AT
1246	2523	TT
1247	2520	TT
1248	2521	TT
1249	2509	TT
1250	2511	TT
1251	2512	TT
1252	2508	TT
1253	2510	TT
1254	2460	AT
1255	2451	AT
1256	2459	TT
1257	2455	AT
1258	2456	AA
1259	2454	AA
1260	2453	AT
1261	2458	AT
1262	2457	TT
1263	2452	TT
1264	2450	AT
1265	2461	AA

Lab #	Wing Band #	Genotype
1266	2434	AT
1267	2437	AT
1268	2422	AT
1269	2431	AT
1270	2424	TT
1271	2430	AT
1272	2435	TT
1273	2427	AA
1274	2436	AA
1275	2432	TT
1276	2425	AT
1277	2438	AT
1278	2426	AT
1279	2428	AT
1280	2429	AA
1281	2433	AT
1282	2423	AA
1283	2415	AT
1284	2419	AT
1285	2416	TT
1286	2418	TT
1287	2420	AA
1288	2421	AT
1289	2417	TT
1290	2371	AA
1291	2377	AA
1292	2368	AA
1293	2375	AT
1294	2373	AT
1295	2370	AT
1296	2374	AT
1297	2372	AT
1298	2376	AT
1299	2369	TT
1300	2363	TT
1301	2358	TT
1302	2364	AT
1303	2359	AT
1304	2365	AT
1305	2361	TT
1306	2360	TT
1307	2366	AT
1308	2367	TT

Lab #	Wing Band #	Genotype
1309	2362	TT
1310	2320	TT
1311	2326	TT
1312	2319	AT
1313	2329	TT
1314	2324	TT
1315	2331	AT
1316	2328	AT
1317	2332	AT
1318	2325	AT
1319	2322	AT
1320	2327	TT
1321	2321	AT
1322	2333	TT
1323	2330	AT
1324	2323	AT
1325	2288	AT
1326	2286	AA
1327	2289	TT
1328	2290	AT
1329	2280	AA
1330	2283	TT
1331	2285	AT
1332	2287	TT
1333	2282	AT
1334	2284	AT
1335	2281	AT
1336	2258	AT
1337	2260	AT
1338	2257	TT
1339	2262	TT
1340	2256	AA
1341	2264	TT
1342	2261	TT
1343	2263	TT
1344	2259	AT
1345	2255	AT
1346	2221	AT
1347	2220	AT
1348	2219	AT
1349	2224	AT
1350	2227	TT
1351	2228	TT

Lab #	Wing Band #	Genotype
1352	2223	AT
1353	2230	AA
1354	2225	AA
1355	2222	TT
1356	2229	AT
1357	2226	TT
1358	2182	AA
1359	2166	TT
1360	2174	TT
1361	2177	AT
1362	2176	TT
1363	2181	AT
1364	2170	TT
1365	2167	TT
1366	2180	AT
1367	2165	AT
1368	2171	AT
1369	2175	TT
1370	2178	AT
1371	2179	AA
1372	2168	TT
1373	2169	AA
1374	2173	AT
1375	2172	AT
1376	2130	AA

Lab #	Wing Band #	Genotype
1377	2137	AT
1378	2131	AT
1379	2135	AA
1380	2133	AT
1381	2134	AA
1382	2128	AT
1383	2136	AT
1384	2132	TT
1385	2129	AT
1386	2126	AT
1387	2127	AT
1388	2051	AT
1389	2052	AT
1390	2055	AA
1391	-	AT
1392	-	TT
1393	-	AA
1394	-	AT
1395	-	AT
1396	-	TT
1397	-	AT
1398	-	TT
1399	-	AT
1400	-	AT

.

APPENDIX D

Second-generation Trial Trimethylamine Data

Wing Band #	Cage #	Geno-type	Diet	Yolk TMA (µg/g)			CV	Serum TMA (µg/ml)
				Egg1	Egg2	Egg3		
2286	241	AA	0	0.98	0.78	1.68	41.21	0.189
2271	242	AA	0	1.70	0.84	3.17	61.90	0.120
1864	371	AA	0	2.59	0.97	1.26	86.38	0.103
2115	372	AA	0	0.88	1.87	3.75	47.44	.
1892	373	AA	0	4.94	7.95	2.99	60.59	0.189
2429	380	AA	0	1.67	0.91	1.19	65.90	0.155
2492	368	AT	0	1.42	1.62	2.25	84.39	0.137
2585	369	AT	0	1.48	1.19	3.02	34.28	0.189
2103	370	AT	0	5.45	4.18	6.08	63.16	.
2180	377	AT	0	2.84	0.86	4.84	28.84	0.103
2104	378	AT	0	1.55	0.73	0.91	51.28	0.240
2212	379	AT	0	5.96	1.22	4.04	56.44	0.189
1953	365	TT	0	1.47	3.87	.	22.86	.
1994	366	TT	0	5.80	7.35	.	6.39	0.155
1997	367	TT	0	1.70	1.85	3.98	30.53	0.137
2526	374	TT	0	1.29	1.02	3.45	43.85	0.155
1862	375	TT	0	1.22	1.04	1.41	57.24	0.000
2158	376	TT	0	0.66	4.92	5.82	65.99	0.223
2581	258	AA	6	1.31	1.27	3.16	33.82	0.189
2179	259	AA	6	1.24	1.26	1.82	.	0.172
2497	260	AA	6	1.59	1.40	1.52	33.82	.
1923	261	AA	6	5.47	6.87	3.63	35.34	0.155
2385	262	AA	6	1.37	1.83	3.16	24.08	0.206
2316	263	AA	6	1.37	4.39	5.55	17.50	.
2197	249	AT	6	2.38	1.04	6.54	10.99	0.155
2014	250	AT	6	1.14	1.83	3.01	44.97	0.189
2105	251	AT	6	1.06	1.21	2.96	35.77	0.137
2113	264	AT	6	1.26	2.10	4.64	22.44	0.206
2265	265	AT	6	1.40	2.08	2.82	68.04	0.137
1901	266	AT	6	.	.	.	16.77	.
2327	252	TT	6	5.73	1.01	4.25	57.17	0.120
2569	253	TT	6	5.35	1.12	1.69	.	0.137
1951	254	TT	6	2.00	3.28	.	12.63	0.189
1977	255	TT	6	5.72	1.40	3.19	9.32	0.137

Wing Band #	Cage #	Geno-type	Diet	Yolk TMA (µg/g)			CV	Serum TMA (µg/ml)
				Egg1	Egg2	Egg3		
1867	256	TT	6	.	1.21	1.83	102.46	0.206
2025	257	TT	6	3.82	1.24	4.03	49.35	0.155
2088	350	AA	12	1.06	1.32	1.60	65.34	0.155
2464	351	AA	12	0.96	1.16	1.07	20.36	0.206
2157	352	AA	12	1.34	2.07	1.60	9.42	0.155
2204	356	AA	12	5.40	4.68	1.09	22.16	0.120
2230	357	AA	12	4.56	1.06	3.40	40.05	0.206
2071	358	AA	12	1.09	0.97	1.32	25.14	0.015
2178	341	AT	12	3.52	1.40	1.02	83.82	0.189
2322	342	AT	12	1.21	1.57	1.69	62.01	.
2396	343	AT	12	1.37	4.89	5.57	59.30	0.086
2530	347	AT	12	1.09	1.42	6.69	15.79	0.137
2562	348	AT	12	1.16	2.25	3.40	63.56	0.137
2590	349	AT	12	1.22	1.70	4.06	16.67	0.120
2443	344	TT	12	7.58	.	.	50.81	.
1824	345	TT	12	2.96	3.68	3.75	24.57	0.292
1820	346	TT	12	4.86	4.48	4.03	51.86	0.172
2263	353	TT	12	13.05	5.95	14.03	18.48	0.155
2013	354	TT	12	3.07	1.85	2.38	53.77	0.275
2379	355	TT	12	6.61	2.36	1.21	67.28	0.189
2099	1307	AA	18	0.94	1.97	1.06	47.21	0.155
2547	1308	AA	18	3.16	2.91	.	69.37	.
1857	1309	AA	18	0.86	1.83	1.22	15.12	0.155
1886	1316	AA	18	1.12	0.88	1.22	72.53	0.086
2405	1317	AA	18	1.39	0.96	1.11	69.91	0.189
2134	1318	AA	18	1.44	1.17	1.37	40.53	0.103
1873	1301	AT	18	4.94	1.21	6.63	63.75	0.120
2276	1302	AT	18	2.64	1.69	1.17	30.59	0.155
2475	1303	AT	18	2.83	0.91	1.48	48.07	0.189
1912	1310	AT	18	1.14	0.45	1.67	51.53	0.103
2466	1311	AT	18	2.23	0.97	1.42	56.21	0.120
2546	1312	AT	18	0.89	1.85	3.11	54.11	0.052
2168	1304	TT	18	5.44	3.29	18.16	54.43	0.275
2407	1305	TT	18	5.11	1.34	8.01	125.65	0.206
1940	1306	TT	18	7.09	4.73	8.61	115.85	0.292
1962	1313	TT	18	7.86	3.39	4.69	68.23	0.206
2208	1314	TT	18	3.52	3.44	8.56	122.13	0.549
2401	1315	TT	18	2.30	1.87	2.08	115.93	0.240
2244	279	AA	24	1.17	2.41	2.40	65.10	0.223
2182	280	AA	24	2.15	2.15	3.11	40.66	0.103
2499	1285	AA	24	1.34	1.72	3.25	56.67	0.120
2256	1292	AA	24	1.49	1.27	4.06	89.66	0.206

Wing Band #	Cage #	Geno-type	Diet	Yolk TMA (µg/g)			CV	Serum TMA (µg/ml)
				Egg1	Egg2	Egg3		
2201	1293	AA	24	1.29	1.16	10.01	69.39	0.086
2339	1294	AA	24	1.17	11.81	.	28.71	0.137
2332	273	AT	24	3.29	1.67	3.26	42.56	0.172
2205	274	AT	24	3.72	4.03	6.84	5.82	.
2319	275	AT	24	1.98	2.66	1.67	37.62	0.103
2554	1289	AT	24	5.27	1.44	4.13	56.30	0.189
2336	1290	AT	24	0.45	7.62	.	41.46	0.137
2455	1291	AT	24	1.31	1.24	9.00	57.10	0.292
1939	276	TT	24	9.62	7.58	10.81	43.27	0.206
1854	277	TT	24	5.49	4.76	4.44	56.70	0.155
1968	278	TT	24	8.87	4.59	.	10.32	0.120
2198	1286	TT	24	8.54	5.07	14.66	16.28	.
2606	1287	TT	24	6.16	5.62	14.36	18.92	0.240
2363	1288	TT	24	8.03	3.59	3.22	10.56	0.155
1858	243	TT	0.11	1.44	3.47	.	58.47	2.454
1991	244	TT	0.11	1.44	1.07	.	20.85	1.257
2500	245	TT	0.11	0.89	1.55	.	38.25	1.223
2435	246	TT	0.11	1.02	1.70	1.83	28.68	1.520
2446	247	TT	0.11	1.70	0.99	1.21	27.96	1.300
2203	248	TT	0.11	1.65	1.02	1.32	23.69	1.333
2408	267	TT	0.055	1.19	1.60	2.97	48.55	1.921
1947	268	TT	0.055	1.21	5.68	3.45	64.85	3.448
1933	269	TT	0.055	2.15	6.13	3.47	51.76	3.916
2621	270	TT	0.055	0.99	1.52	1.70	26.30	1.404
1850	271	TT	0.055	1.59	6.69	9.81	68.82	5.260
2521	272	TT	0.055	1.93	3.39	1.95	34.55	2.423
2002	359	TT	0.165	6.05	4.56	1.14	64.27	3.916
2601	360	TT	0.165	1.06	1.29	3.98	76.95	2.109
2531	361	TT	0.165	2.28	3.65	1.26	50.04	2.396
2262	362	TT	0.165	3.92	5.82	.	27.59	4.866
2246	363	TT	0.165	1.49	1.48	0.74	34.78	1.237
2310	364	TT	0.165	1.19	0.96	1.31	15.42	1.151
1961	1295	TT	0.22	2.40	2.08	10.18	93.87	4.885
2508	1296	TT	0.22	1.44	1.17	1.17	12.37	1.261
2079	1297	TT	0.22	5.73	1.31	13.93	91.61	6.989
2386	1298	TT	0.22	5.35	1.17	3.04	65.71	3.189
2351	1299	TT	0.22	2.31	1.39	3.77	48.20	2.489
1818	1300	TT	0.22	7.15	1.12	3.02	81.92	3.767

.

APPENDIX E

Second-generation Trial Production Data

Cage #	Geno-type	Diet	Hen Days (egg)	Total Eggs	HD Production	Initial Wt (kg)	End Wt (kg)	Hen Days (wt)	ADG (g/d)
241	AA	0	19	19	1.00	2.24	2.21	26.00	-2.31
242	AA	0	19	15	0.79	2.37	2.28	26.00	-0.38
243	TT	0.11	19	15	0.79	2.51	2.45	26.00	-1.15
244	TT	0.11	19	17	0.89	1.87	1.86	26.00	1.54
245	TT	0.11	19	10	0.53	1.43	1.46	26.00	-1.54
246	TT	0.11	19	17	0.89	1.97	1.94	26.00	-2.31
247	TT	0.11	19	16	0.84	2.31	2.37	26.00	2.31
248	TT	0.11	19	15	0.79	2.73	2.67	26.00	-0.77
249	AT	6	19	17	0.89	2.10	2.16	26.00	2.31
250	AT	6	19	14	0.74	2.43	2.33	26.00	-3.85
251	AT	6	19	12	0.63	2.28	2.31	26.00	1.15
252	TT	6	19	19	1.00	1.86	1.85	26.00	-0.38
253	TT	6	19	15	0.79	1.99	1.97	26.00	-0.77
254	TT	6	19	16	0.84	1.52	1.38	26.00	-5.38
255	TT	6	19	14	0.74	1.94	1.87	26.00	-2.69
256	TT	6	19	16	0.84	2.31	2.22	26.00	-3.46
257	TT	6	19	18	0.95	2.43	2.43	26.00	0.00
258	AA	6	19	15	0.79	2.33	2.26	26.00	-2.69
259	AA	6	19	17	0.89	2.06	1.93	26.00	-5.00
260	AA	6	19	17	0.89	1.83	1.79	26.00	-1.54
261	AA	6	19	18	0.95	2.40	2.24	26.00	-6.15
262	AA	6	19	16	0.84	1.98	1.98	26.00	0.00
263	AA	6	19	16	0.84	2.53	2.57	26.00	1.54
264	AT	6	19	19	1.00	2.02	2.01	26.00	-0.38
265	AT	6	19	18	0.95	2.64	2.72	26.00	3.08
266	AT	6	15	13	0.87	2.42	2.30	22.00	-5.45
267	TT	0.055	19	18	0.95	2.00	1.95	26.00	-1.15
268	TT	0.055	19	16	0.84	2.37	2.32	26.00	-3.46
269	TT	0.055	19	17	0.89	1.91	2.03	26.00	-7.69
270	TT	0.055	19	17	0.89	1.97	1.96	26.00	-2.31
271	TT	0.055	19	18	0.95	2.08	2.13	26.00	1.92
272	TT	0.055	19	19	1.00	1.93	1.95	26.00	-1.15
273	AT	24	19	18	0.95	2.31	2.27	26.00	-1.54
274	AT	24	19	17	0.89	2.64	2.52	26.00	-4.62

Cage #	Geno-type	Diet	Hen Days (egg)	Total Eggs	HD Prod-uction	Initial Wt (kg)	End Wt (kg)	Hen Days (wt)	ADG (g/d)
275	AT	24	19	15	0.79	2.37	2.56	26.00	7.31
276	TT	24	19	16	0.84	1.88	1.95	26.00	2.69
277	TT	24	19	14	0.74	1.83	1.88	26.00	1.92
278	TT	24	19	15	0.79	2.12	2.07	26.00	-1.92
279	AA	24	19	16	0.84	2.60	2.52	26.00	-3.08
280	AA	24	19	11	0.58	1.98	2.06	26.00	3.08
341	AT	12	19	16	0.84	2.08	2.08	26.00	0.00
342	AT	12	19	18	0.95	2.43	2.37	26.00	-2.31
343	AT	12	19	15	0.79	2.37	2.28	26.00	-3.46
344	TT	12	6	5	0.83	2.35	2.34	6.00	-1.67
345	TT	12	19	15	0.79	1.80	1.78	26.00	-0.77
346	TT	12	19	16	0.84	2.11	2.06	26.00	-1.92
347	AT	12	19	18	0.95	2.22	2.24	26.00	0.77
348	AT	12	19	16	0.84	2.65	2.46	26.00	-7.31
349	AT	12	19	16	0.84	2.02	2.05	26.00	1.15
350	AA	12	19	18	0.95	2.24	2.15	26.00	-3.46
351	AA	12	19	18	0.95	2.53	2.35	26.00	-6.92
352	AA	12	19	17	0.89	1.51	1.56	26.00	1.92
353	TT	12	19	11	0.58	1.82	1.91	26.00	3.46
354	TT	12	19	18	0.95	2.14	2.24	26.00	3.85
355	TT	12	19	19	1.00	2.34	2.37	26.00	1.15
356	AA	12	19	15	0.79	1.84	1.85	26.00	0.38
357	AA	12	19	18	0.95	2.66	2.68	26.00	0.77
358	AA	12	19	14	0.74	2.42	2.44	26.00	0.77
359	TT	0.165	19	19	1.00	2.17	2.25	26.00	-1.92
360	TT	0.165	19	16	0.84	1.75	1.70	26.00	-3.85
361	TT	0.165	19	18	0.95	2.32	2.27	26.00	1.92
362	TT	0.165	19	15	0.79	2.31	2.26	26.00	5.77
363	TT	0.165	19	15	0.79	2.54	2.48	26.00	-11.15
364	TT	0.165	19	19	1.00	2.06	2.06	26.00	-3.08
365	TT	0	19	12	0.63	2.37	2.32	26.00	-4.62
366	TT	0	19	12	0.63	2.28	2.18	26.00	-7.31
367	TT	0	19	7	0.37	2.29	2.34	26.00	3.85
368	AT	0	19	18	0.95	2.76	2.73	26.00	3.08
369	AT	0	19	16	0.84	2.21	2.25	26.00	-1.92
370	AT	0	19	16	0.84	2.56	2.52	26.00	-1.92
371	AA	0	19	18	0.95	2.18	1.98	26.00	1.15
372	AA	0	19	14	0.74	1.69	1.63	26.00	-1.15
373	AA	0	19	15	0.79	2.10	2.15	26.00	2.31
374	TT	0	19	18	0.95	2.61	2.76	26.00	-2.31
375	TT	0	19	10	0.53	1.97	1.68	26.00	3.85
376	TT	0	19	16	0.84	2.80	2.72	26.00	6.54

Cage #	Geno-type	Diet	Hen Days (egg)	Total Eggs	HD Prod-uction	Initial Wt (kg)	End Wt (kg)	Hen Days (wt)	ADG (g/d)
377	AT	0	19	15	0.79	2.26	2.20	26.00	-1.92
378	AT	0	19	15	0.79	2.15	2.21	26.00	-2.31
379	AT	0	19	17	0.89	1.77	1.75	26.00	0.00
380	AA	0	19	11	0.58	2.99	2.96	26.00	-2.31
1285	AA	24	19	14	0.74	1.65	1.68	26.00	1.15
1286	TT	24	19	15	0.79	2.50	2.39	26.00	-4.23
1287	TT	24	19	16	0.84	1.87	1.98	26.00	4.23
1288	TT	24	19	17	0.89	2.46	2.46	26.00	0.00
1289	AT	24	19	14	0.74	1.68	1.72	26.00	1.54
1290	AT	24	19	6	0.32	2.07	2.12	26.00	1.92
1291	AT	24	19	16	0.84	1.76	1.82	26.00	2.31
1292	AA	24	19	16	0.84	1.84	1.96	26.00	4.62
1293	AA	24	19	19	1.00	2.05	2.13	26.00	3.08
1294	AA	24	19	10	0.53	2.13	2.08	26.00	-1.92
1295	TT	0.22	19	17	0.89	2.06	1.94	26.00	-1.92
1296	TT	0.22	19	17	0.89	2.15	1.96	26.00	-1.92
1297	TT	0.22	19	14	0.74	2.68	2.78	26.00	4.62
1298	TT	0.22	19	15	0.79	2.20	2.14	26.00	-0.38
1299	TT	0.22	19	15	0.79	2.42	2.52	26.00	1.92
1300	TT	0.22	19	17	0.89	2.19	2.36	26.00	0.77
1301	AT	18	19	14	0.74	2.24	2.36	26.00	4.62
1302	AT	18	19	18	0.95	1.79	1.85	26.00	2.31
1303	AT	18	19	18	0.95	2.56	2.38	26.00	-6.92
1304	TT	18	19	14	0.74	1.64	1.74	26.00	3.85
1305	TT	18	19	16	0.84	2.13	2.16	26.00	1.15
1306	TT	18	19	16	0.84	1.85	1.89	26.00	1.54
1307	AA	18	19	15	0.79	1.93	1.89	26.00	-1.54
1308	AA	18	8	6	0.75	2.12	2.04	8.00	-10.00
1309	AA	18	19	17	0.89	2.50	2.50	26.00	0.00
1310	AT	18	19	17	0.89	2.53	2.47	26.00	-2.31
1311	AT	18	19	19	1.00	2.27	2.19	26.00	-3.08
1312	AT	18	19	12	0.63	2.07	2.13	26.00	2.31
1313	TT	18	19	15	0.79	1.90	1.97	26.00	2.69
1314	TT	18	19	17	0.89	2.16	2.18	26.00	0.77
1315	TT	18	19	19	1.00	2.32	2.29	26.00	-1.15
1316	AA	18	19	16	0.84	1.94	2.00	26.00	2.31
1317	AA	18	19	14	0.74	2.09	2.03	26.00	-2.31
1318	AA	18	19	18	0.95	2.74	2.55	26.00	-7.31

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APPENDIX F

Other Strain Genotypes

Lohmann Brown		Lohmann WLH		ISA Brown	
Lab #	**Genotype**	**Lab #**	**Genotype**	**Lab #**	**Genotype**
9013	AA	11	AA	8801	AA
9019	AT	12	AA	8802	AA
9027	AT	16	AA	8805	TT
9028	AT	18	AA	8806	AT
9029	AA	30	AA	8811	AA
9030	AT	31	AA	8812	TT
9032	AA	33	AA	8813	AT
9033	AA	40	AA	8814	AA
9034	AA	46	AA	8815	AT
9036	AT	56	AA	8816	AA
9041	AA	62	AA	8817	AT
9043	AA	66	AA	8818	AT
9045	AT	68	AA	8819	TT
9046	AA	71	AA	8823	AT
9048	AT	76	AA	8826	AA
9052	AT	78	AA	8827	AA
9053	AA	89	AA	8828	AA
9054	AT	93	AA	8830	TT
9055	AT	103	AA	8832	TT
9057	AA	135	AA	8833	TT
9058	AA	149	AA	8834	AA
9059	AT	176	AA	8837	AA
9060	AA	180	AA	8839	AA
9062	AT	202	AA	8840	AT
9063	AT	206	AA	8842	AA
9068	AT	220	AA	8843	TT
9069	AT	223	AA	8844	AT
9071	AA	228	AA	8849	AA
9073	AT	240	AA	8852	AT
9075	AT	261	AA	8857	AT
9076	AT	262	AA	8858	AA
9078	AT	268	AA	8859	AA
9081	AA	278	AA	8860	AT
9087	AT	287	AA	8861	AT
9088	AA	289	AA	8862	AT

Lohmann Brown		Lohmann WLH		ISA Brown	
Lab #	**Genotype**	**Lab #**	AA	**Lab #**	**Genotype**
9089	AA	308	AA	8866	TT
9092	AT	309	AA	8867	AT
9093	AT	318	AA	8869	AT
9096	AA	319	AA	8875	AA
9098	AT	325	AA	8887	TT
9099	AA	331	AA	8888	AA
9108	AA	331	AA	8889	AA
9114	AT	333	AA	8890	AT
9115	AA	360	AA	8892	AT
9119	AT	369	AA	8893	AT
9120	AT	389	AA	8894	AA
9122	AT	409	AA	8897	AT
9123	AA	428	AA	8898	AT
9124	AA	452	AA	8903	AA
9125	AA	457	AA	8905	AT
9126	AT	458	AA	8909	AT
9129	AA	466	AA	8910	TT
9133	AT	531	AA	8911	AA
9134	AT	534	AA	8912	AA
9138	AA	539	AA	8916	AT
9139	AT	542	AA	8917	AA
9140	AA	547	AA	8918	AA
9141	AT	559	AA	8920	AA
9142	AA	571	AA	8923	AT
9145	AT	581	AA	8924	AT
9148	AA	593	AA	8925	AT
9149	AT	611	AA	8927	AT
9151	AA	619	AA	8930	AT
9154	AA	622	AA	8932	AA
9155	AT	626	AA	8933	AT
9157	AA	634	AA	8934	AA
9158	AT	639	AA	8935	AA
9160	AA	662	AA	8936	AT
9162	AA	663	AA	8937	AA
9164	AT	680	AA	8938	AT
9165	AT	701	AA	8946	AA
9166	AA	714	AA	8952	AA
9167	AT	721	AA	8957	AA
9169	AT	725	AA	8965	AA
9170	AA	743	AA	8969	TT
9171	AT	750	AA	8970	AT
9174	AA	752	AA	8973	AT
9175	AT	760	AA	8978	AT

Lohmann Brown		Lohmann WLH		ISA Brown	
Lab #	**Genotype**	**Lab #**	**Genotype**	**Lab #**	**Genotype**
9177	AT	777	AA	8980	AA
9180	AA	794	AA	8981	AA
9181	AT	807	AA	8983	AA
9183	TT	826	AA	8985	AA
9185	AA	829	AA	8986	AA
9186	AA	840	AA	8989	TT
9188	AT	845	AA	8996	AT
9189	AT	847	AA	8997	AA
9191	AT	859	AA	9000	AT
9192	AT	861	AA	9001	AT
9195	AT	878	AA	9007	TT
9197	AA			9012	AT
9198	AA			9013	AA
9200	AA			9019	AT
9203	AT			9025	AA
9205	AA				
9209	AA				
9211	AT				
9213	AT				
9215	AT				
9220	AA				
9223	AT				

APPENDIX G

WPC Abstract

XXIII World's Poultry Congress, June 30-July 4, 2008, Brisbane, Australia.

Dietary and genetic interactions in fishy egg tainting in brown-shelled layers

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Fishy egg tainting is a problem associated with feeding canola meal (CM) to brown-shelled laying hens. It is caused by a single nucleotide polymorphism (A to T transversion) in flavin containing monooxygenase 3 (FMO3), which leads trimethylamine accumulation (TMA) in the yolk. The purpose of this study was to characterize the effects of FMO3 genotype and level of dietary CM on egg TMA, as well as to observe daily variation in egg TMA for individual hens. Commercial laying hens were genotyped at FMO3, with 43 AT (heterozygous) and 41 TT (homozygous for tainting) hens used in this trial. Approximately 10 hens of each genotype were allocated to one of four dietary treatments: 0, 6, 12, or 18% CM. Diets were fed for 3 weeks prior to collecting 3 eggs per hen for TMA analysis. Level of CM did not affect yolk TMA content in AT hens with mean values of 3.53, 2.34, 2.29 and 2.90 μg/g for 0, 6, 12, 18% CM inclusion, respectively. In contrast, yolk TMA increased linearly (2.58, 4.50, 6.52, 8.03 μg/g) with diet CM level for TT hens. Significant variation ($P<0.05$) was found in yolk TMA levels for eggs derived from the same hen. These data confirm the recessive nature of the T mutation and indicate that limits for dietary CM are not required for AT hens.

APPENDIX H

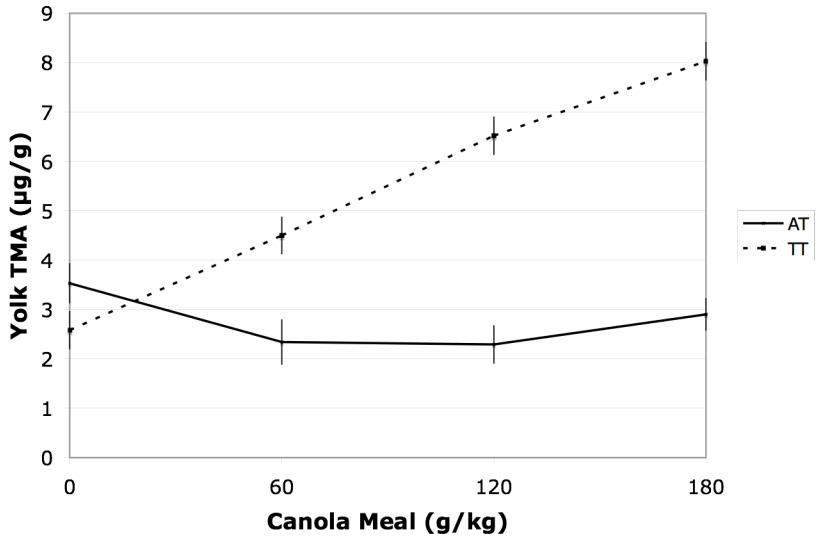
WPC Short Paper

XXIII World's Poultry Congress, June 30-July4, 2008, Brisbane, Australia.

DIETARY AND GENETIC INTERACTIONS IN FISHY EGG TAINTING IN BROWN-SHELLED LAYERS

A.K. WARD, H.L. CLASSEN AND F.C. BUCHANAN

Fishy egg tainting is a problem associated with feeding canola meal (CM) to brown-shelled laying hens. It is caused by an A to T transversion at nucleotide 1043 in *flavin containing monooxygenase 3* (*FMO3 g1043A>T*), which leads to trimethylamine accumulation (TMA) in the yolk (Honkatukia *et al.*, 2005). The purpose of this study was to characterize the effects of FMO3 genotype and level of dietary CM on egg TMA, as well as to observe daily variation in egg TMA for individual hens. Commercial laying hens were genotyped at *FMO3 g1043A>T*, with 43 AT (heterozygous) and 41 TT (homozygous for tainting) hens used in this trial. Approximately 10 hens of each genotype were allocated to one of four dietary treatments: 0, 60, 120, or 180 g/kg CM. Diets were fed for 3 weeks prior to collecting 3 eggs per hen for TMA analysis. Data were analyzed as a two by four factorial design using the mixed procedure of SAS version 9.1, with $P<0.05$ as significant. Means were separated using Tukey's Honestly Significant Difference Test. The relationship between level of diet CM and egg TMA content were also examined within genotypes using the correlation procedure of SAS.



Significant variation (P=0.0033) was found between yolk TMA levels for eggs derived from the same hen. The effects of diet, genotype, and their interaction were highly significant (P<0.0001). There was a significant linear correlation between yolk TMA and dietary CM for the TT hens (P<0.0001, R^2=0.64) but not for the AT hens. It can therefore be concluded that CM inclusion does not cause egg tainting in hens of the AT genotype for *FMO3 g1043A>T*. These data confirm the recessive nature of the T mutation and indicate that limits for dietary CM are not required for AT hens.

Honkatukia, M., Reese, K., Preisinger, R., Tuiskula-Haavisto, M., Weigend, S., Roito, J., Mäki-Tanila, A. and Vilkki, J. (2005). *Genomics*, **86**:225-232.

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APPENDIX I

ISAG Abstract

International Society for Animal Genetics XXXI, July 20-24, 2008, Amsterdam, The Netherlands.

Fishy-egg tainting by brown-shelled layers is recessively inherited under typical commercial conditions

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Fishy-egg taint has long been a problem associated with feeding canola meal (CM) to brown-shelled laying hens. It is caused by a SNP in the *flavin-containing monooxygenase 3* gene (*FMO3 984c.A>T*). This mutation prevents the fishy-smelling trimethylamine (TMA) from being oxidized to the non-odourous trimethylamine *N*-oxide (TMAO), leading to an accumulation of TMA in developing egg yolks. TMA is produced from the bacterial fermentation of choline in the gut. Conflicting results from previous studies have found egg tainting to be recessive or additive. Both studies fed high concentrations of choline chloride to induce tainting, which does not reflect commercial production practices. Our objective was to characterize the inheritance pattern of fishy-egg tainting when hens are fed CM, reflecting typical industry practices. Diets consisting of 0, 6, 12, 18, or 24% CM were fed to 6 hens per genotype per diet (n=90). Three eggs were collected per hen and the yolks were analyzed for TMA concentration. The effects of diet, genotype, and their interaction were all significant ($P<0.0001$). Only hens of the TT genotype displayed increasing yolk TMA concentration with increasing CM. We therefore conclude that fishy-egg tainting is recessively inherited under standard CM feeding practices.

2052

Fishy-egg Tainting by Brown-shelled Layers is Recessively Inherited Under Typical Commercial Conditions

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Introduction

The production of fishy-tainted eggs has long been associated with feeding canola meal to brown shelled laying hens[1]. The choline in canola meal is fermented by enteric bacteria to the fishy-smelling compound trimethylamine (TMA), which is then absorbed into circulation[2]. Under normal circumstances the TMA is metabolized by flavin-containing monooxygenase 3 (FMO3) to the non-odourous trimethylamine *N*-oxide and excreted in the urine[3]. However, a SNP in *FMO3* (*FMO3 c.984A>T*) renders FMO3 unable to metabolize TMA[4]. Subsequently TMA accumulates in circulation and is deposited in developing egg yolks. The human detection threshold for TMA in whole egg is estimated to be 1 µg/g[5], which is equivalent to approximately 4 µg/g in yolk. Our objective was to determine the inheritance pattern of fishy-egg taint when hens are fed canola meal at and above levels typically used in commercial egg production.

Materials and Methods

- Birds of a commercial brown-shelled strain were bred to produce hens for this trial
- The hens were genotyped for *FMO3 c.984A>T* by PCR-RFLP
  - Forward = GCT CAT CAC CCG CTT CTG G
  - Reverse = GCC TCG TTG TTC TTG CTT TCG
  - *BsrI* cuts "A" allele
- 6 hens per genotype were assigned to one of 5 dietary treatments: 0, 6, 12, 18, or 24% canola meal
- Hens were fed the diets for 3 weeks prior to the collection of 3 eggs per hen for yolk TMA analysis[6]
- Data were analyzed as a 3 x 5 factorial design using the mixed procedure of SAS 9.1.Significance was set at P<0.05
- Linear regression analysis performed separately for each genotype to examine the response in yolk TMA concentration to increasing levels of canola meal

Results

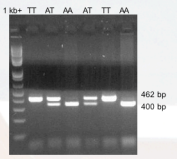

Figure 1. Agarose gel of *FMO3 c.984A>T* PCR-RFLP products.

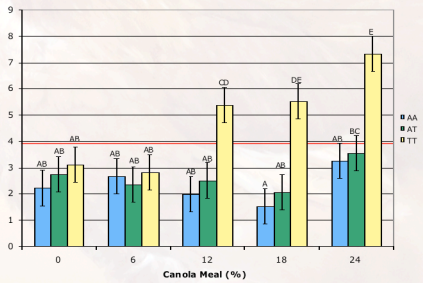

Figure 2. Interaction between yolk TMA concentration and canola meal inclusion level for each *FMO3 c.984A>T* genotype. Red line represents human detection threshold in yolk. Means with the same letter at not significantly different (P<0.05).

Table 1. Linear regression between yolk TMA concentration and canola meal level for each *FMO3 c.984A>T* genotype

Genotype	P Value	R^2 Value
AA	0.6358	0.0080
AT	0.4144	0.0248
TT	**0.0005**	**0.3599**

Discussion

- There was no difference in yolk TMA concentration between the AA and AT genotypes within any of the diets
- No change in yolk TMA concentration with increasing levels of canola meal was observed in hens of the AA and AT genotypes
- In both the AA and AT genotypes, mean yolk TMA concentration remained below the human detection threshold for all of the diets
- There was a significant increase in yolk TMA concentration with increasing levels of canola meal for hens of the TT genotype
- TT hens produced tainted eggs at canola meal levels of 12% and higher

Conclusion

Fishy-egg tainting is recessively inherited when hens are fed canola meal at levels typical of commercial egg production

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Hy-Line