A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet

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Abstract

A significant number of genetically modified (GM) crops have been approved to enter human food and animal feed since 1996, including crops containing several GM genes 'stacked' into the one plant. We randomised and fed isowean pigs (N=168) either a mixed GM soy and GM corn (maize) diet (N=84) or an equivalent non-GM diet (N=84) in a long-term toxicology study of 22.7 weeks (the normal lifespan of a commercial pig from weaning to slaughter). Equal numbers of male and female pigs were present in each group. The GM corn contained double and triple-stacked varieties. Feed intake, weight gain, mortality and blood biochemistry were measured. Organ weights and pathology were determined post-mortem. There were no differences between pigs fed the GM and non-GM diets for feed intake, weight gain, mortality, and routine blood biochemistry measurements. The GM diet was associated with gastric and uterine differences in pigs. GM-fed pigs had uteri that were 25% heavier than non-GM fed pigs (p=0.025). GM-fed pigs had a higher rate of severe stomach inflammation with a rate of 32% of GM-fed pigs compared to 12% of non-GM-fed pigs (p=0.004). The severe stomach inflammation was worse in GM-fed males compared to non-GM fed males by a factor of 4.0 (p=0.041), and GM-fed females compared to non-GM fed females by a factor of 2.2 (p=0.034).

Key words: GMO**,** GM corn, GM soy, GM animal feed, toxicology, stomach inflammation, uterus weight.

Introduction

Genetically modified (GM) crops have entered human food and animal feed in increasing amounts since they were commercially released into fields in the USA in 1996 (USDA, 2011). The main traits in GM crops to date have been to express proteins for herbicide tolerance (Ht) and insect resistance (Carman, 2004; USDA, 2011). Herbicide tolerant crops are engineered to produce one or more proteins that allow the crop to survive being sprayed with a given herbicide. Insect resistant crops are usually engineered to produce

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one or more insecticidal proteins that are toxic to target insects. The latter proteins are usually Bt proteins, so named because they are structurally similar to naturally-occurring Cry proteins from a soil bacterium, *Bacillus thuringiensis* (ANZFA, NDb). Hence these crops are also called Bt crops.

Of the GM crops planted in the USA, herbicide-tolerant GM soy has been widely adopted and now constitutes 94% of the soy planted in the USA (USDA, 2011). GM corn varieties have also been widely adopted in the USA (USDA, 2011). They usually contain Ht or Bt traits, or a 'stacked' combination of them (Pioneer Hi-Bred, 2012).

Prior to the release of a new GM crop into the food supply, the developer provides food regulators in many countries with studies it has done on the crop. These studies often include animal feeding studies, even though some regulators, such as Australia's, do not require them (FSANZ, ND; Carman, 2004), while the USA has a voluntary system. Many food regulators do not require any studies to be done on crops containing several "stacked" genes if all the genes in the stack have previously been individually approved for use in the same kind of plant (EFSA, 2010; FSANZ, 2010). Consequently, safety studies on stacked crops are less frequent, even though an analysis of official data (USDA, 2011) indicates that over 37% of GM corn varieties currently planted in the USA are stacked with both Ht and Bt traits.

There have been a number of reviews of the published literature on the safety of GM crops. For example, Flachowsky et al. (2005) and Preston (2005) both conducted reviews and both concluded that GM crops were safe for animals and people to eat. However, many of the feeding studies reviewed used non-mammals (e.g. birds, fish) or animals were fed the crop in a form that humans do not eat (e.g. silage) or only animal production outcomes were measured such as body weight, carcass weight, breast meat yield or milk production, which may not be indicative of potential human health outcomes (Carman, 2004). Only a small proportion of published animal feeding studies have been longer-term toxicological studies where a GM crop was fed to animals that are physiologically comparable to humans, and organs, blood and tissue samples were taken from the animals and examined to assess if the crop caused any adverse effects.

Two recent reviews of these rarer toxicology-type studies have recently been published. Snell et al. (2011) reviewed 12 studies of 90 days or longer duration and concluded that GM plants were nutritionally equivalent to non-GM plants and could be safely used in food and feed. However, once again, most of the studies reviewed used animals that were either not physiologically comparable to humans, or used only small numbers of animals. A broader picture is given in a series of three reviews by Domingo (2000; 2007) and Domingo & Bordonaba (2011). The first two papers concluded that there were few published studies investigating toxicology or health risks, while the third found that most of the more recent studies concentrate on only a few GM crops (soy, corn and rice), ignoring many other GM crops such as potatoes, peas and tomatoes.

Another review of 19 studies of mammals fed GM soy or maize has recently been conducted (Séralini et al., 2011). These authors also reviewed the raw data of some other authors' 90-day feeding studies. They found some evidence for adverse liver and kidney effects from eating some GM crops and concluded that 90-day feeding studies were insufficient to evaluate chronic toxicity of GM crops.

More recently, a highly publicised (e.g. Poulter, 2012), much longer study of two-years' duration on NK603 herbicide-tolerant corn (which contains one of the genes fed in the present study) has been published (Séralini et al. 2012). There were indications of higher death rates, more tumours and liver and kidney pathologies in GM-fed rats.

The aim of the present study was to perform a thorough, long-term toxicology study (for 22.7 weeks, being the normal lifespan of a commercial pig from weaning to slaughter) on pigs in a USA commercial piggery in order to compare the effects of eating either a mixed GM soy and GM corn diet, or an equivalent diet with non-GM ingredients. Pigs in the USA are usually fed a mixed corn and soy diet, containing a high proportion of GM varieties. Even though pigs are physiologically similar to humans, particularly for gastrointestinal observations, very few toxicology studies have been conducted on them for GM crops (Walsh et al., 2012a). In doing this study, we not only used animals that were physiologically similar to humans, but we also weighed and internally examined organs and took blood for biochemical analysis. We further used a large enough sample size (168 pigs, 84 per group) to be able to determine statistical significance for key toxicological outcomes. We also used GM crops that are planted in significant quantities in the USA (Ht soy, and Ht and Bt corn) and hence are commonly eaten by pigs and humans in the USA. We further fed these crops as a mixed diet. Mixed diets commonly occur for pigs and humans. This study therefore reflects the effects of eating GM crops in the 'real world'. To our knowledge, this is the first study of its kind conducted.

Materials and Methods

Animal feed

In accordance with usual commercial USA piggery practice, soy and corn were obtained direct from farmers who had grown it commercially. Different GM corn varieties are usually co-mingled in farm storage. The corn used in this study contained 90% DK 42-88 RR YG PL (a triple stack of NK603, MON863 and MON810 genes) with the remainder being equal quantities of Pannar 5E-900RR (containing NK603), Pannar 4E-705RR/Bt (a double stack of NK603 and MON810) and Producers 5152 RR (containing NK603). Therefore, the GM corn that was used was genetically modified to produce three new proteins. Two were Bt proteins that protected the plant against insect attack, while the third protein provided the plant with tolerance to the herbicide glyphosate (Testbiotech, 2012; Monsanto, 2012).

Because Roundup Ready™ (RR) soy is predominant in the GM soy market, this was used. This crop contains a gene that provides tolerance to the herbicide glyphosate. GM DNA analysis (Genetic ID, Fairfield, Iowa, US) confirmed that the GM corn contained a combination of NK603, MON863 and MON810 genes (expressing the CP4 EPSPS, Cry 3Bb1 and Cry 1Ab proteins respectively), that the RR soy was 100% RR soy (expressing the CP4 EPSPS protein), that the non-GM feed contained a median of 0.4% GM corn and that the non-GM soy contained a median of 1.6% GM soy. Such GM contamination of apparent non-GM material is common in the US.

In a similar way to the GM crops used, non-GM soy and non-GM corn were also obtained direct from farmers who had grown it commercially for human food and animal feed. Isogenic parental varieties of the GM crops, from which the GM crops were developed, were not used because they are generally not commercially available to buy. Furthermore, triple-stacked corn containing all three genes used here was developed

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from conventionally cross-breeding several GM crops, each of which has a non-GM parent, leading to a multiplicity of isogenic parental varieties that would need to be used in combination for a control diet. As the aim of this study was to compare the effects of GM and non-GM varieties present in animal feed and human food in the real world, the soy and corn for the control diet was instead chosen as a mixture of non-GM soy and corn that was destined for animal feed and human food and that came from the same geographical area. The GM soy and corn used in this study have been determined to be compositionally and substantially equivalent to non-GM varieties of soy and corn by government regulators (ANZFA, 2002, NDa, NDb; FSANZ, 2003, 2006) which indicates that there should be no phenotypical variation between the GM and non-GM varieties used in this study that could influence the outcomes measured in this study.

GM and non-GM corn were both ground using the same cleaned equipment, size screen and revolutions per minute to obtain the same particle size. GM and non-GM soy beans were also processed on the same type of cleaned equipment - using Insta-Pro extruders and expellers, rather than being solvent-extracted, in order to preserve the identity of the beans during processing into soybean meal. This process purees the beans and squeezes out most of the oil, leaving a residual oil content of 8%. In the process, the beans are heated to 153^{o}C to 166^{o}C. As pigs grow, they require different amounts of nutrients, so six different sub-diets were progressively used. Soy content decreased from 26.5% to 13.0%, corn increased from 56.4% to 83.8% and protein decreased from 18.3% to 13.3% of the diet (Table 1). Ingredients, including supplements, were those routinely used by the piggery and were the same between groups. The GM and non-GM diets had the same protein, energy, macro- and micro-nutrient contents and only differed in the use of GM or non-GM soy and corn. Pigs were fed on a self-feeding, full-feed basis. The amount of feed consumed by each group was recorded.

Table 1. Details of the six body-weight-specific sub-diets used progressively as pigs grew.

	Sub-diet number					
	1	**2**	**3**	**4**	**5**	**6**
Pig weight (lb)[a]	14-25	25-60	60-90	90-130	130-200	200-260
No. days on diet[b]	39-40	17-18	23-24	24-25	37-38	15-17
Average daily intake (lb)	0.9	2.43	3.45	4.71	6.10	6.78
Protein (%)	18.6	18.0	17.4	16.3	15.2	14.7
Soy (%)[c]	26.5	25.0	23.4	20.4	17.5	16.0
Corn (%)[d]	70.0	71.6	73.2	76.3	79.8	81.3
UN premix (%)[e]	2.5	2.5	—	—	—	—
UG premix (%)[f]	—	—	2.5	2.5	—	—
UF premix (%)[g]	—	—	—	—	2.5	2.5
Boost premix (%)[h]	0.0025	0.0025	0.001	0.0015	0.0015	0.0015
Extra lysine	—	—	0.001	0.0005	—	—
Extra $CaCO_3$ (%)	0.0075	0.0075	0.006	0.006	0.002	0.002
200 mesh bentonite clay (%)	0.0035	0.0035	0.0035	0.0035	0.0035	0.0035

a As the piggery was in the USA, pig diets were changed when pigs reached a certain weight in pounds.

b Because pig handlers were required to keep to usual piggery practices and were blinded as to the GM feeding status of each group of pigs, pigs in each group were changed from one sub-diet to the next according to the body weight of the group. Consequently, one group was often changed to the next sub-diet a day before the other group. While the GM-fed group spent one day longer on a particular diet than the non-GM group for three diets, the non-GM group spent a day longer on a particular diet for the other three diets. Therefore, there was neither a trend nor a difference in the progression of the two groups from one diet to another. Pigs were fed for a total of 158 days if they were slaughtered on the first of the two slaughter days, and 159 days if they were slaughtered on the second slaughter day.

c GM soy went into the GM diets and non-GM soy into the non-GM diets.

d GM corn went into the GM diets and non-GM corn into the non-GM diets.

e Ultra Nursery Plus Premix from Advanced Biological Concepts, Osco, Illinois, containing (as copied directly from the label) guaranteed amounts of 0.5% crude protein, 6.0% lysine, 0.5% crude fat, 3.0% crude fiber 13.0% to 15% calcium, 13.0% phosphorus, 16.0% to 18.0% sodium chloride, 10ppm selenium, 1,500 ppm zinc, 190,000 IU/lb vitamin A, 25,000 IU/lb vitamin D_3 and 800 IU/lb vitamin E. Other ingredients on the label (not quantified), include: copper, iron, zinc, manganese, choline, ascorbic acid, niacin, riboflavin, pantothenic acid, vitamin K, vitamin B_{12}, carotene and iodine.

f Ultra Grower Premix Plus from Advanced Biological Concepts, Osco, Illinois, containing (as copied directly from the label) guaranteed amounts of 0.5% crude protein, 1.0% lysine, 0.5% crude fat, 3.0% crude fiber, 15.0% to 17% calcium, 12.0% phosphorus, 15.0% to 17.0% sodium chloride, 3ppm selenium, 1,500 ppm zinc, 160,000 IU/lb vitamin A, 22,000 IU/lb vitamin D_3 and 800 IU/lb vitamin E. Other ingredients on the label (not quantified) include: copper, iron, zinc, manganese, choline, niacin, riboflavin, pantothenic acid, vitamin K, vitamin B_{12}, carotene and iodine.

g Ultra Finisher Premix Plus from Advanced Biological Concepts, Osco, Illinois, containing (as copied directly from the label) guaranteed amounts of 0.5% crude protein, 3.0% lysine, 0.5% crude fat, 3.0% crude fiber, 18.0% to 20.0% calcium, 10.0% phosphorus, 6.5% to 7.5% sodium chloride, 3ppm selenium, 4,000 ppm zinc, 125,000 IU/lb vitamin A, 20,000 IU/lb vitamin D_3 and 500 IU/lb vitamin E. Other ingredients on the label (not quantified) include: copper, iron, zinc, potassium, magnesium, manganese, choline, ascorbic acid, niacin, riboflavin, pantothenic acid, vitamin K, vitamin B_{12}, carotene and iodine.

h Natural Boost from Advanced Biological Concepts, Osco, Illinois, containing (as copied directly from the label) guaranteed amounts of 10.0% crude protein, 0.005% lysine, 0.005% methionine, 1.0% crude fat, 24.0% crude fiber, 40.0% acid detergent fiber, 0.2% to 0.7% calcium, 0.2% phosphorus, 1.0% to 1.5% sodium chloride, 0.5% potassium, 500ppm copper, 1,500 ppm zinc, 180,000 IU/lb vitamin A, 55,000 IU/lb vitamin D_3 and 500 IU/lb vitamin E. Other ingredients on the label (not quantified) include: iron, zinc, magnesium, manganese, choline, cobalt, ascorbic acid, niacin, riboflavin, pyridoxine HCl, pantothenic acid, biotin, vitamin K, vitamin B_{12}, folic acid, carotene and iodine.

Mycotoxin analyses (Midwest Laboratories Inc, Omaha, Nebraska, US) showed 2.08 ppb total aflatoxins and 3.0 ppm total fumonisins in a pooled sample of the GM feed and no aflatoxins and 1.2 ppm total fumonisins in a pooled sample of the non-GM feed. No other mycotoxins were detected. These levels are well below the USA and EU limits for mycotoxins in pig feed. In addition, according to common industry practice, a mycotoxin binding agent (200 mesh bentonite clay) was added to the diets of young pigs (Table 1).

Animals

Standard commercial Yorkshire-cross piglets were obtained from a commercial farrowing facility as a result of crossing Hampshire Duroc males with Yorkshire Landrace females. All sows were fed the same diet containing some GM ingredients and were impregnated at a similar time to obtain isowean piglets. Male piglets were neutered at three days of age in order to fulfill market requirements for meat free of boar-taint.

Unweaned piglets (N=168; average 24 days of age) were transported to the piggery nursery and randomly placed into pens of 14 each. Pens were then randomly allocated to receive either a GM or non-GM diet. Animals were weighed and then fed their allocated diet as their first solid food. After 32 days, pigs were transported to a different facility for the 'growing and finishing' phase, where they continued on their allocated diets but were housed as 42 pigs per pen with outside access. Throughout, pigs were housed according

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to usual industry practices, under shelter on concrete floors. They experienced the natural daytime/night-time temperature and light/dark cycle.

Data collected from live pigs

Individual weights were recorded weekly and animals were monitored daily by observers who were blinded to a pig's dietary group. Daily measurements included inside and outside air temperature, air quality, weather conditions, level of activity of pigs around the feeder and the appearance of the feeder itself, the level of activity of the pigs around the water and the appearance of the water, details of any pigs found dead, details of any pigs that were moved away from, or back to, the 'home pen' and the reasons for this (e.g. they were being harassed by other pigs), level of contentment (measured as content, irritable or aggressive), presence of cough or sneeze, the presence of any skin problems (e.g. pale or discoloured skin or the presence of rashes or sores), any eye problems, and the consistency of the stools (normal, some loose or runny stools, lots of loose or runny stools). Blood was taken from the jugular vein of awake pigs according to standard industry methods two days before the first pigs were slaughtered. The blood was taken from a random subset of pigs in the following pattern to prevent any time-related bias: approx. half the pigs in the non-GM-fed group, approx half the pigs in GM-fed group, the remainder of the non-GM-fed group, and the remainder of the GM-fed group. Blood was centrifuged and serum was removed and frozen. Blood biochemical analyses were undertaken by Marshfield Clinic Laboratories, Marshfield, WI, USA, who were blinded to all aspects of the study. The laboratory's reference range for awake three to four month-old Yorkshire cross pigs was used as it was most applicable for this study.

Autopsy procedure

When the pigs were 26 weeks old, they were fasted for 18 hours and transported to a large commercial abattoir where they were slaughtered according to the usual, approved methods of the abattoir on two consecutive days. On each day, approximately equal numbers of GM-fed and non-GM-fed pigs were slaughtered to prevent any temporal between-group bias. Pigs on each day were killed within a few minutes of each other. The internal organs were carefully removed to prevent faecal contamination and placed in individual identified buckets with 2 litres of cold phosphate-buffered saline to quickly chill the organs. Organs were kept under near-freezing conditions until they were examined by two licenced, practicing veterinarians with considerable porcine experience. They were blinded as to which pigs were fed GM feed. To remove any between-inspector bias, one veterinarian examined all the kidneys, hearts, lungs and stomachs while the other examined all the livers, spleens, intestines, uteri and ovaries. Veterinarian comments and organ weights were recorded by the same person to remove any between-person measurement bias or recording bias. Where evisceration resulted in incomplete removal of an organ, veterinarians determined if disease had caused part of an organ to adhere to the chest or abdominal wall and hence remain with the carcass, as well as the nature of that disease. The weights of partial organs were not included in statistical analyses due to the errors they would have produced. Kidney weights were the sum of both kidneys per pig. Ovary weights were the sum of both ovaries per pig except for two GM-fed pigs where one ovary was accidentally removed by the abattoir. Here, the weight of both ovaries was estimated by doubling the weight of the remaining ovary. Intestines could not be weighed or inspected due to the amount of digesta still present in them, even after 18 hours of fasting, so the external surface of the intestines was examined for abnormalities

and any intramural, palpable tissue masses. Organ weights were analysed as a percentage of body weights.

In addition to externally examining the organs, veterinarians also examined the interior of every kidney using a single, deep transverse cut, every heart by slicing into both ventricles and both atria, and every lung using at least two deep cuts through the dorsal surface of each lung lobe, and if abnormalities were found, several more cuts to properly identify the abnormality and its extent. Every stomach was examined by cutting it open along the length of its greatest curvature, washing out the contents and inspecting the entire internal surface of the opened-flat stomach, including rugae.

Data analysis

A stomach erosion was defined as an abnormal stomach surface that had a visible area of current inflammation and oedema and where the mucosa was starting to separate (and which could potentially progress to form an ulcer). The length of any ulcer was measured in millimetres. If an ulcer had a clot in it, or showed frank bleeding, it was recorded as a bleeding ulcer. If an ulcer was less than 1 mm in length, it was recorded as a pin-point ulcer, otherwise as a frank ulcer. When calculating the total length of ulceration in each stomach in mm, each pin-point ulcer was numerically rounded to be 1mm in length. Stomach inflammation was scored by the attending, blinded veterinarian as a result of expertise obtained from numerous pig autopsies and a classification system developed as a result of an earlier, preliminary study on pig stomachs. These stomachs were obtained from a random sample of pigs from the same abattoir and came from pigs raised by other commercial pig producers. Inflammation was classified as nil, mild, moderate, or severe based on a combination of the area of current inflammation and level of redness and swelling. Typical examples of each of the four categories of inflammation are shown in Figure 1. For a severe level of inflammation, almost the whole fundus had to be swollen and cherry-red in colour.

Data were analysed using the statistical packages SPSS and EpiInfo. Continuous data were analysed by removing SPSS-identified extreme outliers, being those more than three times the interquartile range away from the lower or upper quartiles. This conservative and well-established approach better tests the nature of the underlying distribution. Data were then tested for normal distribution using the Shapiro-Wilk test. If a normal distribution was found for both dietary groups, data were expressed as means and standard deviations and were analysed using parametric methods (t-test), otherwise data were expressed as medians and ranges and analysed using non-parametric methods (Mann-Whitney U test). Categorical data were analysed using uncorrected chi-squared tests unless an expected cell value was less than five, when Fisher's Exact was used.

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Figure 1. Different levels of stomach inflammation found (clockwise from top left): nil (from a non-GM-fed pig, number B41), mild (from a non-GM-fed pig, number B15), moderate (from a GM-fed pig, number C34) and severe (from a GM-fed pig, number D22).

Results

There were no statistically significant differences in food intake, feed conversion ratios, number or nature of illnesses, number or nature of veterinary interventions, veterinary costs or mortality between the non-GM-fed and GM-fed groups of pigs. Mortalities were 13% and 14% for the non-GM-fed and GM-fed groups respectively, which are within expected rates for US commercial piggeries. All dead pigs were autopsied by blinded veterinarians and deaths were assessed as due to usual commercial piggery-related matters and not to their diets. There was also no difference in body weights between the two dietary groups, initially, during, or at the end of the experiment. Initial weights in kg were : non-GM-fed group: 6.71 + 1.05 (mean + standard deviation); GM-fed group: 6.87 + 0.97. Final weights were: non-GM-fed group: 100.42 + 22.84; GM-fed group: 101.75 + 21.92.

Autopsy results

Organ weights were not statistically different between GM-and non-GM-fed pigs except for uterine weights (Table 2). After removing one extreme outlier, the medians of the non-GM-fed (now N=33) and GM-fed (N=37) groups became 0.084% and 0.105% of the body weight respectively. That is, the median uterus weight of GM-fed pigs, as a proportion of

body weight, was 25% higher than that of non-GM-fed pigs, which was statistically significant (p=0.025).

There was no difference in the disease status of organs between the two groups of pigs except for the level of inflammation in the stomachs of the pigs (Table 3, Figure 1). For non-GM-fed pigs, stomach inflammation was concentrated in the mild and moderate range, whereas GM-fed pigs showed much more severe inflammation (p=0.004). GM-fed pigs showed severe stomach inflammation at a rate of 2.6 times that of non-GM-fed pigs (95% confidence interval = 1.29-5.21) (Table 3). This occurred in both male (p=0.041) and female (p=0.034) pigs (Table 4). We found severe stomach inflammation in 22.2% of male pigs fed the GM diet and in 41.7% of female pigs fed the GM diet (compared to 5.6% and 18.9%, respectively, in pigs fed the non-GM diet (Table 4).

Blood biochemistry

Blood biochemistry results are given in Table 5. Aspartate transaminase (AST), potassium and creatine kinase (CK) were not statistically analysed because they were raised substantially in both dietary groups due to the way blood was collected and hence they were unable to reflect any effect of feeding a GM diet. AST and potassium were raised because the collection needle was pushed through muscle, while CK was raised due to the pigs being alert and restrained while blood was taken. While bicarbonate can increase if pigs pant or squeal unduly during blood taking, no pigs recorded a bicarbonate concentration higher than the reference range (Table 6), so this variable was retained in analyses.

To determine if feeding the GM diet was associated with a clinically abnormal biochemistry profile, the proportion of pigs in each dietary group that lay above (or below) the reference (normal) range were then compared (Table 6). No statistically significant differences were found. The means or medians of the biochemical variables were also compared. No significant differences were found (Table 5).

The analyses of several biochemical variables were confounded by the level of haemolysis in the blood sample. Haemolysis can be a problem when taking blood from alert animals, and in non-laboratory settings due to lag times between sampling and centrifuging blood. Haemolysis was reported as nil, mild, moderate or severe by the laboratory. Total bilirubin, urea nitrogen, creatinine, phosphorus, calcium, sodium, chloride, bicarbonate, and anion gap were found to be significantly correlated with the level of haemolysis (results not shown) and hence haemolysis was regarded as a confounder for these variables. Spearman's rho test was used as a measure of the association rather than the Pearson correlation co-efficient as it is less sensitive to outliers and does not assume normality. These biochemical variables then underwent multiple linear regression to control for the effect of haemolysis. As known confounders should be controlled-for, even if they do not appear as actual confounders in initial studies, glucose also underwent this process. No biochemical variable was found to have a significant relationship to the diet with the level of haemolysis controlled-for (results not shown). Consequently, no biochemical differences were found between non-GM-fed and GM-fed pigs. However, the concentration of GGT, which is a measure of liver heath, was 16% lower in GM-fed pigs than non-GM-fed pigs and this result was on the borderline of statistical significance (Table 5).

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Table 2. Organ weights (as a percentage of body weight) - descriptive statistics of raw data and statistical comparisons of extreme outlier-removed data.

	Non-GM-fed						GM-fed						Statistical comparison of dietary groups	
	n[a]	Mean	SD[b]	Median	Min	Max	n[a]	Mean	SD[b]	Median	Min	Max	Test used[c]	p[d]
Kidneys	66	0.32	0.066	0.31	0.19	0.66	68	0.33	0.057	0.32	0.16	0.56	t	0.51
Heart	69	0.40	0.065	0.40	0.27	0.63	69	0.41	0.059	0.40	0.27	0.61	MW	0.79
Liver	71	1.81	0.342	1.77	1.27	3.20	72	1.79	0.348	1.71	1.25	3.16	MW	0.45
Spleen	73	0.16	0.033	0.16	0.11	0.33	71	0.16	0.032	0.15	0.093	0.30	t	0.40
Lung	67	0.91	0.241	0.87	0.58	2.00	68	0.98	0.315	0.94	0.57	2.52	MW	0.20
Stomach	73	0.62	0.130	0.57	0.42	0.99	71	0.64	0.129	0.60	0.44	1.01	MW	0.26
Uterus	34	0.10	0.048	0.086	0.040	0.31	37	0.12	0.053	0.105	0.036	0.244	MW	0.025*
Ovaries	36	0.0085	0.0027	0.0081	0.0040	0.019	36	0.0086	0.0023	0.0084	0.0047	0.014	t	0.38

a An organ was not included in the analysis if adhesions caused only a partial organ to remain with the viscera, due to the errors inclusion would have caused.
b Standard deviation
c After tests for normality, groups were compared by 2-tailed t-test if data from both dietary groups were normally distributed, Mann Whitney U test (MW) otherwise.
d* p<0.05 to 0.01, ** p<0.01 to 0.001, *** p<0.001

Table 3. The proportion of pigs in each dietary group with adverse findings on gross pathology

Organ	Condition	Proportion with condition				Relative risk of condition in GM-fed pigs	95% confidence interval of the relative risk	p[a]
		Non-GM-fed		GM-fed				
		No. N=73	%	No. N=72	%			
Kidney	Any abnormality	0	0.0	0	0.0	—[b]	—[b]	—[b]
Heart	Any abnormality[c]	11	15.1	5	6.9	0.46	0.17-1.26	0.119
Liver	Any abnormality[d]	6	8.2	3	4.2	0.51	0.13-1.95	0.494
Spleen	Any abnormality[e]	3	4.1	2	2.8	0.68	0.12-3.93	1.000
Lung	Pneumonia[f]	42	57.5	43	59.7	1.04	0.79-1.36	0.789
	Fibrous pleuritis or pericarditis	9	12.3	4	5.6	0.45	0.15-1.40	0.153
	Abnormal lymph nodes[g]	13	17.8	16	22.2	1.25	0.65-2.40	0.506
Stomach	Nil inflammation	4	5.4	8	11.1	2.03	0.64-6.44	0.218
	Mild inflammation	31	42.5	23	31.9	0.75	0.49-1.16	0.190
	Moderate inflammation	29	39.7	18	25.0	0.63	0.39-1.03	0.058
	Severe inflammation	9	12.3	23	31.9	2.59	1.29-5.21	0.004**
	Erosion(s)	63	86.3	58	80.6	0.93	0.81-1.08	0.352
	Pin-point ulcer(s)	13	17.8	9	12.5	0.70	0.32-1.54	0.373
	Frank ulcer(s)	15	20.5	17	23.6	1.15	0.62-2.12	0.657
	Bleeding ulcer(s)	0	0.0	2	2.8	—[b]	—[b]	0.245
Intestines	Any abnormality	0	0.0	0	0.0	—[b]	—[b]	—[b]
Uterus	Filled with fluid[h]	0[i]	0.0	2[j]	5.6	—[b]	—[b]	0.493
Ovary	Any abnormality	0[k]	0.0	0[l]	0.0	—[b]	—[b]	—[b]

a Uncorrected chi-square test unless an expected cell value was less than five, when Fisher exact test (2-tailed) was used. * p<0.05 to 0.01, ** p<0.01 to 0.001, *** p<0.001
b No statistic could be calculated because one or more cells contained zeros.
c Adhesions and/or fibrous pericarditis and/or scar tissue.
d Adhesions and/or fibrinous tags and/or the presence of fibrin.
e Adhesions and/or fibrinous tags.
f Consolidating bronchopneumonia of the cranial ventral lung lobe(s) and/or caudal lobe(s).
g Haemorrhagic and/or swollen bronchial lymph node(s).
h When two uteri were removed from neighbouring organs, fluid oozed from them.
i N=36. Of 37 females, one had a congenital defect. It had only the beginnings of a uterine tract and no uterus or ovaries.
j N=36.
k N=36. Of 37 females, one had a congenital defect. It had only the beginnings of a uterine tract and no uterus or ovaries.
l N=35. Of 36 females, one had a uterus but no ovaries, which were removed by accident during slaughter and retained by the slaughterhouse.

Table 4. Stomach inflammation by gender.

Gender	Level of stomach inflammation	Proportion with condition				Relative risk of condition in GM-fed pigs	95% confidence interval of the relative risk	p[a]
		Non-GM-fed		GM-fed				
		No.[b]	%	No.[c]	%			
Males	Nil	1	2.8	4	11.1	4.00	0.47-34.07	0.357
	Mild	16	44.4	12	33.3	0.75	0.42-1.35	0.334
	Moderate	17	47.2	12	33.3	0.71	0.40-1.26	0.230
	Severe	2	5.6	8	22.2	4.00	0.91-17.56	0.041*
Females	Nil	3	8.1	4	11.1	1.37	0.33-5.70	0.711
	Mild	15	40.5	11	30.6	0.75	0.40-1.41	0.373
	Moderate	12	32.4	6	16.7	0.51	0.22-1.22	0.118
	Severe	7	18.9	15	41.7	2.20	1.02-4.76	0.034*

a Uncorrected chi-square test unless an expected cell value was less than five, when Fisher exact test (2-tailed) was used. * p<0.05 to 0.01, ** p<0.01 to 0.001, *** p<0.001
b N=36 for males, N=37 for females.
c N=36 for males, N=36 for females.

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Table 5. Blood biochemistry descriptive statistics of raw data and statistical comparisons of extreme outlier-removed data.

	Non-GM-fed			GM-fed			Reference range[a]		Statistical comparison of dietary groups	
	N	Median[b] (Mean)	Range[b] (SD)	N	Median[b] (Mean)	Range[b] (SD)	Standard (asleep)[c]	Awake (Yorkshire X)[d]	Test used[e]	p[f]
Glucose (mg/dL)	39	89.0	58 – 109	38	90.5	52 – 111	85 – 150	58.0 – 197.0	MW	0.81
AST[g](U/L)	39	60.0	21 – 2757	38	57.0	12 – 1724	32 – 84	0.0 – 45.0	MW	0.72
Total bilirubin (mg/dL)	39	0.10	0.1 – 0.3	38	0.10	0.1 – 0.3	0.0 – 1.0	0.1 – 0.2	MW	0.76
Cholesterol (mg/dL)	39	100.0	56 – 140	38	100.0	55 – 125	36 – 54	50.0 – 92.0	MW	0.85
Total protein (g/dL)	39	(6.48)	(0.95)	38	(6.63)	(0.91)	7.9 – 8.9	5.1 – 6.9	t	0.16
Albumin (g/dL)	39	4.00	1.7 – 4.7	38	4.10	1.7 – 4.8	1.9 – 3.3	3.0 – 4.4	MW	0.59
Urea nitrogen (mg/dL)	39	11.0	5 – 22	38	12.0	8 – 29	10 – 30	4.3 – 12.7	MW	0.30
Creatinine (mg/dL)	39	0.90	0 – 1	38	0.70	0 – 1	1.0 – 2.7	0.9 – 1.9	MW	0.21
Phosphorus (mg/dL)	39	(9.1)	(1.5)	38	(9.1)	(1.5)	5.3 – 9.6	6.2 – 9.2	t	0.99
Calcium (mg/dL)	39	10.70	5.5 – 11.3	38	10.50	5.1 –12.0	7.1 –11.6	9.1 – 10.8	MW	0.94
Sodium (mmol/L)	37	140.0	98 – 148	37	140.0	98 – 145	135 - 150	132.0–144.0	MW	0.60
Potassium (mmol/L)	38	6.35	4.6 – 13.9	37	6.40	4.3 –16.3	4.4 – 6.7	3.4 – 5.0	MW	0.56
Chloride (mmol/L)	38	97.0	67 – 104	37	98.0	66 – 102	94 – 106	94.0 – 103.0	MW	0.86
Bicarbonate (mmol/L)	39	33.0	19 – 37	38	33.5	18 – 37	18 – 27	28.0 – 37.0	MW	0.44
CK[h] (U/L)	39	2416.0	214 –22500	38	1960.0	10 –22500	61 –1251	264.0–1247.0	MW	0.73
GGT[i](U/L)	39	(35.1)	(18.4)	38	(29.5)	(18.1)	10 – 60	0.0 – 60.0	t	0.05
Anion gap (mmol/L)[j]	37	16.0	12 – 23	37	15.0	11 – 27	–	–	MW	0.61

a From Marshfield Clinic, Marshfield, WI, USA.

b Medians and ranges are reported for non-parametric comparisons, means and standard deviations for parametric comparisons.

c Marshfield Clinic's usual reference range. Pigs were anaesthetised to obtain blood.

d Marshfiled Clinic's reference range for awake, 3-4 month-old Yorkshire cross pigs. This was used as it is much more applicable to this study.

e After tests for normality, groups were compared by two-tailed t-test if data from both dietary groups were normally distributed, Mann Whitney U test (MW) otherwise.

f * p<0.05 to 0.01, ** p<0.01 to 0.001, *** p<0.001

g Aspartate transaminase.

h Creatine kinase.

i Gamma-glutamyl transferase.

j There is no laboratory reference range for anion gap. Sorbitol dehydrogenase results were not given by the lab on this occasion.

Table 6. Biochemical variables compared to the reference range[a] to determine clinical significance.

Biochemical variable	Number (%) above or below reference range			
	Non-GM-fed (N=39)		GM-fed (N=38)	
	Above reference range	Below reference range	Above reference range	Below reference range
Glucose	0 (0)	0 (0)	0 (0)	2 (5)
AST[b]	23 (59)	—[c]	24 (63)	—[c]
Total bilirubin	1(3)	0 (0)	1 (3)	0 (0)
Cholesterol	29 (74)	0 (0)	28 (74)	0 (0)
Total protein	10 (26)	4 (10)	17 (45)	3 (8)
Albumin	7 (18)	5 (13)	3 (8)	5 (13)
Urea nitrogen	10 (26)	0 (0)	16 (42)	0 (0)
Creatine	0 (0)	18 (46)	0 (0)	23 (61)
Phosphorus	12 (31)	2 (5)	16 (42)	1 (3)
Calcium	10 (26)	9 (23)	14 (37)	6 (16)
Sodium	2 (5)[d]	4 (11)[d]	0 (0)[d]	4 (11)[d]
Potassium	34 (89)[e]	0 (0)[e]	36 (97)[d]	0 (0)[d]
Chloride	1 (3)[e]	7 (18)[e]	0 (0)[d]	4 (11)[d]
Bicarbonate	0 (0)	5 (13)	0 (0)	5 (13)
CK[f]	24 (62)	2 (5)	27 (71)	1 (3)
GGT[g]	2 (5)	—[c]	1 (3)	—[c]

a Awake Yorkshire cross pig reference range from Marshfield Clinic, Marshfield, WI, USA. Anion gap has no reference range so was not included in the table.
b Aspartate transaminase.
c It was not possible for a pig to record a concentration below the bottom of the reference range, which was zero.
d N=37.
e N=38.
f Creatine kinase.
g Gamma-glutamyl transferase.

Discussion

In this study, we found that female pigs fed the GM diet had median uterine weights that were 25% greater than non-GM-fed pigs (p=0.025). This result is attributed to the difference in diet as other variables were controlled for, including the presence of mycotoxins, and possible confounders such as infectious diseases, animal husbandry considerations and various forms of bias such as temporal, between-person, measurement or recording bias, as these were all controlled-for. The concentration of mycotoxins in the feed was insignificant, both dietary groups received the same nutrients and care, the care complied with industry standards, and all those doing laboratory analyses and weighing, caring for, slaughtering and doing autopsies on pigs were blinded as to the dietary group of each pig.

The reported difference in uterine weight warrants further investigation in future studies because such a biologically significant difference in uterine weights may reflect endometrial hyperplasia or carcinoma, endometritis, endometriosis, adenomyosis, inflammation, a thickening of the myometrium, or the presence of polyps. The uteri from two GM-fed pigs were full of fluid compared to nil from non-GM-fed pigs (Table 3) which may be linked to pathology. The link between an increase in uterine weights and GM feeding is supported by other authors (Brasil et al., 2009) who found that GM soy-fed rats had a statistically significant 59% increase in the density of the uterine endometrial glandular epithelium compared to rats fed an equivalent organic soy diet. Further studies should include histology, blood oestrogen, progesterone and cytokine concentrations, and which GM crop(s) and their GM protein products may, or may not, be involved. As this study used neutered males, further studies are required to investigate any potential effect of these crops on male reproduction. Multigenerational reproductive studies should also be considered.

In this study, a diet of GM feed had no effect on stomach erosions or ulceration but had a significant effect on inflammation. Pigs fed the mixed GM soy and GM corn diet showed 2.6 times the rate of severe stomach inflammation compared to non-GM fed pigs. This biologically significant finding was statistically significant ($p=0.004$). GM-fed male pigs showed severe stomach inflammation at a rate of 4.0 times that of the non GM fed male pigs ($p=0.041$); and female pigs showed a rate of severe stomach inflammation that was 2.2 the rate of the non-GM fed female pigs ($p=0.034$).

The pig industry uses finely-ground feed to maximise feed efficiency which can increase inflammation and ulceration of the stomach (Wolf, 2010). We therefore controlled the grind size, removing it as a confounder. Hence our results show that these GM crops were associated with stomach inflammation that was additional to any that may be caused by particle size. The result is attributed to the difference in diet, since the presence of mycotoxins, possible confounders such as infectious diseases, animal husbandry considerations or temporal, between-person, measurement and recording bias were controlled across the two groups.

One explanation for the inflammation results could lie with the Cry 3Bb1 and Cry 1Ab proteins that these GM corn varieties are engineered to produce. They act as insecticides by inducing pore formation and disintegration of the gut tissue (Spok et al., 2007) of certain grubs that attack corn plants. It has been argued that these proteins cannot harm the gastrointestinal tract of mammals because mammals lack the necessary gut environment and receptors (ANZFA, 2000). However, Vazquez-Padron et al. (2000) found six proteins in the mouse small intestine that could bind to a Cry protein (Cry 1Ac). Furthermore, when the Cry protein bound to these proteins, it resulted in hyperpolarisation of the intestine, which is consistent with the formation of cationic channels, as occurs in the insect gut (Vazquez-Padron et al., 2000). In addition, an independent in vivo study found structural changes and hyperplasia in the ileum of mice fed a Cry protein for two weeks (Fares & El-Sayed, 1998). Chowdhury et al. (2003) and Walsh et al. (2012b) found the Cry1Ab protein (which was present in the feed in our study) throughout the digestive tract of pigs. Chowdhury et al. (2003) found the protein (and sections of the gene that codes for it) in the stomach, duodenum, ileum, caecum and rectum of pigs fed Bt11 corn for four weeks, while Walsh et al. (2012b) found the protein in the stomach, caecum and colon of pigs fed MON810 corn for 110 days (they

appear not to have looked in the rectum), indicating that this protein is resistant to digestion in pigs. In our study, stomach inflammation may be due to one or both of the Cry proteins fed in the study and future studies may provide answers.

The findings in this study are conservative since the non-GM diet pigs were exposed, albeit minimally, to potential GMO impacts. The presence of small amounts of GM material in the non-GM feed, using out-bred animals, piglets from GM-fed sows, and performing the study in a commercial setting (including the potential exposure of the pigs to any infectious diseases common to US commercial pigs and taking blood on site) could be expected to reduce any differences between the two dietary groups.

We found that our key findings were not reflected in the standard biochemical tests often undertaken by researchers in this area, probably because such tests provide a poor measure of inflammation and matters associated with uterine size. We suggest that the following may be better measures: the red blood cell count and haematocrit to measure anaemia and iron deficiency from possible blood loss, C-reactive protein and white blood cell count to measure inflammation, and oestrogen and progesterone.

In addition, if an autopsy is done at the end of a GM crop feeding experiment, this often involves only a visual inspection of the exterior of organs without weighing them. However by weighing organs we found a significant 25% increase in uterine weights in the GM-fed pigs. Moreover, where organs are weighed in such studies, they are often not examined internally (Carman, 2004) and such an approach would preclude finding the stomach inflammation reported in the present study.

The present study is an observational study of the action of a mixture of GM crops on the health of pigs, versus a comparable non-GM diet. Future work will investigate individual GM crops, will involve histopathology, and will consider mechanisms for reported group differences.

Conclusion

Pigs fed a GMO diet exhibited heavier uteri and a higher rate of severe stomach inflammation than pigs fed a comparable non-GMO diet. Given the widespread use of GMO feed for livestock as well as humans this is a cause for concern. The results indicate that it would be prudent for GM crops that are destined for human food and animal feed, including stacked GM crops, to undergo long-term animal feeding studies preferably before commercial planting, particularly for toxicological and reproductive effects. Humans have a similar gastrointestinal tract to pigs, and these GM crops are widely consumed by people, particularly in the USA, so it would be be prudent to determine if the findings of this study are applicable to humans.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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