Repercussions of politicized regulation exemplified by compulsory new TC1507-maize 90-day rat feeding study

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Abstract

Politicized and prescriptive regulation of genetically modified (GM) crops has unintended adverse effects, including misdirected resources and reduced benefits. In the case of animal testing, this suboptimal resource use includes needless animal sacrifice. Whole-food animal feeding studies are generally of negligible value in GM crop risk assessment, a position that was affirmed by the European Food Safety Authority (EFSA). Contrary to EFSA's 2011 position, in 2013, the European Commission directed that 90-day rat studies be conducted for new GM events. As no EFSA guidance was available for these studies in a test hypothesis absence, EFSA interpreted this as a mandate to develop a prescriptive study design. Recently, EFSA has retroactively required 90-day rat studies be completed following their new study guidelines for previously approved single events as part of breeding stack approvals. Unable to secure an EFSA derogation, a new compulsory TC1507 maize 90-day rat study was conducted to support a breeding-stack which confirmed the previous study results of TC1507 maize not adversely affecting rats. This politically driven requirement for animal testing is at odds with international standards for animal welfare, provides no scientific value to the GM breeding stack safety assessment and is not proportionate to the potential risk.

Keywords: TC1507, DAS-Ø15Ø7-1, 90-day rat feeding study, regulation, prescriptive, GMO, EFSA

Highlights:

- The European Food Safety Authority (EFSA) oversees the safety assessment of GM crops
- EFSA concluded that 90-day rat feeding studies are generally not required to assess safety
- Despite this, European Regulators require rat feeding studies for all new GM events

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- EFSA developed guidelines for 90-day rat feeding studies that differ from OECD standards
- During the review of stacked products containing single events with studies pre-dating the new guidelines EFSA is requesting that certain studies are repeated
- A repeat 90-day rat feeding study for the widely commercialized product, TC1507 maize, was requested and performed showing that the product is safe to rats
- This retroactively applied requirement for an approved product is not scientifically justified

Introduction

Initial risk assessment for DAS-Ø15Ø7-1 maize: Herculex™ I maize is a genetically modified (GM) insect-resistant and herbicide-tolerant event (DAS-Ø15Ø7-1; hereafter rereferred to as TC1507) expressing the lepidopteran insecticidal Cry1F protein from *Bacillus thuringiensis* (Bt) and the phosphinothricin acetyltransferase (PAT) enzyme from *Streptomyces viridochromogenes*, the latter of which confers tolerance to the herbicidal active ingredient glufosinate (Baktavachalam et al., 2015). TC1507 maize was first commercialized in 2003 and has been approved for food and/or feed use in more than twenty countries, including the European Union (EU) (EFSA, 2004, 2005a, 2005b; ISAAA, 2021). As part of the safety assessment process required by certain regulatory agencies, a 90-day rat feeding study with TC1507 maize grain was completed in 2002. The study followed internationally agreed guidelines (MacKenzie et al., 2007) and was accepted by the European Food Safety Authority (EFSA: regulatory agency responsible for assessing the safety of GM crops in the EU) to grant food and feed approval in the EU in 2006 (Baktavachalam et al., 2015). Since that time, EFSA has published positive scientific opinions for a dozen breeding combinations of TC1507 maize with other approved GM maize events (breeding stacks; **Table 1 in Appendix**) to be as safe as their non-GM comparator(s). Furthermore, a number of livestock feeding studies have confirmed the nutritional wholesomeness of TC1507 maize grain (Baktavachalam et al., 2015; **Table 2 in Appendix**).

Regulatory history of rat studies in the European Union: 90-day rat feeding studies were initially required to investigate potential unintended adverse nutritional or health effects possibly generated from the transformation process (unintended crop-compositional changes) (Herman & Ekmay, 2014). However, research has shown that genetic engineering has a lower potential to unexpectedly alter crop composition compared with traditional breeding (Herman & Price, 2013; Schnell et al., 2015), and that 90-day rat feeding studies would have very low power to detect adverse compositional changes even if they were to occur (Bartholomaeus et al., 2013). Based on the low relative value of 90-day rat feeding studies in assessing the risk of GM crops, EFSA recommended in 2011 that

performance of 90-day rat feeding studies should be reserved for cases where other evidence from the safety assessment suggested potential for the occurrence of an adverse effect (hypothesis-based study design) (EFSA, 2011) as adopted by many other regulatory authorities around the world. However, the European Commission (executive branch of the EU) disagreed with EFSA, and in 2013, obtained agreement from a majority of member states that a 90-day rat study should be conducted for all new GM transformation events (European Commission, 2013). This requirement appeared to be predicated on increasing the public trust in approval decisions (Herman et al., 2021; Gheysen et al., 2019). The European Commission expressed its hopes that performing these studies would impact on the way EU member states consider such products (though no shift in voting behavior has occurred). Rather than accepting the preexisting international standard methods used for 90-day rat feeding studies (OECD, 1998), EFSA interpreted this decision by the European Commission as a *de facto* regulatory requirement for 90-day rat feeding studies for all GM events (EFSA, 2011), and developed a prescriptive method for studies in the absence of a test hypothesis (EFSA, 2014). The overall consequence of this series of events is that EFSA created *de facto* regulatory requirements for a study they initially determined was not scientifically justified on a routine basis (Herman et al., 2021; Devos et al., 2014; Kuiper et al., 2013).

Registration of Breeding Stacks in the European Union: When two or more single previously approved transformation events are combined through traditional breeding (breeding stacks), the EU requires these breeding stacks be approved before use in food and feed. In contrast to countries which have used experience to reduce or eliminate their regulatory requirements for breeding stacks, regulatory requirements for breeding stacks have been steadily increasing in the EU (Bell et al., 2018; Herman et al., 2017; Weber et al., 2012). This scientifically questionable practice was codified into legislation in 2013 (European Commission, 2013). As part of the approval process for breeding stacks, EFSA now requires all component (single) events to be evaluated in 90-day rat feeding studies. Recently, EFSA extended this requirement to include that single-event 90-day rat feeding studies supporting breeding stacks must retroactively comply with the regulatory requirements put in place in 2014 (EFSA, 2018a).

Novel TC1507 rat feeding study: As previously described, a 90-day rat feeding study with TC1507 maize grain was initially completed in 2002 (MacKenzie et al., 2007). This study was conducted in compliance with international guidance (OECD, 1998) and existing regulation (European Commission, 2001) and was consistent with the informal recommendations of the European Commission's Scientific Committee on Plants assessing GMOs (under Regulation 2001/18/EC), specifically, that grain grown for inclusion in the diet not be sprayed with trait-related herbicides to avoid confounding potential herbicide-induced compositional effects

with potential trait-induced effects. This study was evaluated by EFSA in their positive food and feed assessments for TC1507 maize in 2004 and 2005 (EFSA, 2004, 2005a, 2005b). The same study was considered acceptable during the subsequent safety assessments for a dozen breeding stacks containing the TC1507 event (**Table 1 in Appendix**). However, EFSA has recently stated that, for breeding-stack assessments, applicants must now supply a 90-day rat feeding study that complies with the recent regulatory requirements (**Table 3 in Appendix**), even for previously approved single events that have been in commerce for many years (EFSA, 2018a). Despite multiple attempts to obtain agreement with EFSA of the appropriateness of a derogation (exemption), and despite the existing 90-day rat feeding study having previously been considered acceptable by EFSA to conclude that TC1507 maize is as safe as conventional maize, a new 90-day rat feeding study with TC1507 maize grain was required to support authorization of a breeding stack of previously-approved single events in compliance with the *post hoc* regulatory requirements put in place in 2014 (EFSA, 2014). Comparison of the design elements between the original and recent 90-day rat feeding studies are described in **Table 3 in Appendix**. Briefly, the new study included paired housing vs. individual housing used in the original study, larger group sizes of 16/sex vs. 12/sex in the original study, blinding of scientific and technical staff to treatment group and randomized allocation of animals within the study room vs. stratification by group in the original study. A slightly higher maximum incorporation rate was used in the new study (50% vs. 33%). Very few novel safety endpoints were included in the new study, with the most prominent being determination of thyroid hormone values (T3, T4, and TSH) and weights, and inclusion of mammary gland histopathology for male animals. Changes to the statistical analysis for the new study included consideration of the cage as the experimental unit (ExpU), combined analysis of endpoint data across genders, when possible, estimation of standardized effect sizes (SES), and incorporation of adjustments for multiplicity of testing. Herein, the results of this redundant and compulsory new study are summarized, and the consequences of a highly politicized regulatory process and overly prescriptive regulation are discussed.

Materials and Methods

Grain from TC1507 maize treated with glufosinate, control maize and commercial reference maize lines were fully characterized using methods validated in accordance with Good Laboratory Practices (GLPs; US-EPA, 1989) for presence or absence of the event, expressed trait protein concentration and/or composition and contaminant analyses (mycotoxins and pesticide residues). Composition analyses including proximate, fiber, amino acids, minerals, select heavy metals, vitamins, fatty acids, anti-nutrients, and secondary metabolites for all maize grain lots were determined as previously described (Anderson et al., 2019; Cong et al., 2015; Herman & Ekmay, 2014; Malley et al.,

2007). All compositional and contaminant analyses were conducted by EPL Bio Analytical Services, Inc. (EPL-BAS; Niantic, IL). Six experimental rodent diets were formulated to balance crude protein and were manufactured from the five fully characterized maize grain lots by Purina Test Diet (Richmond, IN) based on the profile for PMI Certified Rodent LabDiet® 5002. The maize grain was incorporated at a fixed inclusion rate of 50% by weight. Diet characterization consisted of nutrient composition and contaminant analyses, molecular characterization for presence/absence of the event, and concentration, homogeneity, and stability analyses of the expressed Cry1F protein using validated GLP methods.

The design of eight cages per diet and sex was determined to be sufficient to achieve greater than 80% power to detect the targeted effect size of biological relevance based on the statistical power analyses required by EFSA for 90-day feeding studies with whole genetically modified food and feed (**Figure S1 in Supplementary Information**; Hong et al., 2017). The study was conducted in compliance with GLPs (US-EPA, 1989) at Haskell Global Center for Health Sciences (Newark, DE), an AAALAC-accredited test facility, and the protocol was approved by the Haskell Institutional Animal Care and Use Committee (IACUC). Sprague Dawley [Crl:CD(SD)] rats were obtained from Charles River Laboratories International, Inc (Raleigh, NC), and were received as a single shipment of the same approximate age. Four days prior to initiation of experimental diet administration, animals were assigned to each cage pair within sex based on weight and assigned to blocks based on the mean animal pair body weight and to cage rack positions by sex as previously described (Hong et al., 2017). Diets were randomly assigned to a cage within each block. Cage racks were placed into an animal room which was maintained at 20-25°C and 30-70% relative humidity, with a 12-hour light/dark cycle, and all animals were provided tap water *ad libitum*. During the 7-day acclimation/quarantine period, all animals were fed PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum*. The characterized diets were fed *ad libitum* to the animals for at least 90 consecutive days during the in-life phase of the study. The study design complied with OECD, Section 4 (Part 408) test guideline (OECD, 1998) including selected endpoints new to the test guideline in the 2018 version (OECD, 2018), and with EFSA guidance for 90-day rodent feeding studies (EFSA, 2011; 2014; **Table 3 in Appendix**).

Diet treatment groups included: 1) TC1507 "High" maize incorporated at 50%; 2) TC1507 "Low" maize incorporated at 33% + control (isogenic) maize incorporated at 17% (50% total maize); 3) control (isogenic) maize incorporated at 50%; and three additional non-GM reference diet groups each containing 50% of a different non-GM reference maize grain (P0760, P05089, and XL5840).

For each endpoint, data from test groups fed TC1507 High or TC1507 Low were

statistically compared with the group fed the control maize diet both across gender (when possible) and within each gender via statistical tests associated with an appropriate statistical analysis approach. Data from the reference diets were not included in statistical analyses. The statistical models or methods used depended on the characteristics of each endpoint. Data for some endpoints (e.g., food consumption) were collected or calculated on a per cage basis and were modeled with the experimental unit and observation unit set to the cage. The other endpoints (e.g., body weight) were collected or calculated on an individual rat basis and were modeled considering cage the unit of replication and rat the unit of observation. Continuous endpoints were analyzed using linear mixed models; non-continuous endpoints were analyzed using contingency-table based methods. Endpoints only involving sex-specific organs were analyzed using linear mixed models without gender effects. If no statistical method was appropriate for an endpoint, it was not statistically analyzed. As indicated by EFSA in their scientific opinion for 90-day oral toxicity studies in rodents with whole food and feed (EFSA, 2011), multiplicity due to separate analysis of large number of endpoints was addressed by applying the false discovery rate (FDR) control method (Benjamini & Hochberg, 1995; Westfall et al., 1999). Statistical methods used in this study were previously described in Hong et al. (2017).

Study Results

Molecular evaluation using PCR confirmed the presence of the TC1507 event in the TC1507 diets and the absence of the TC1507 event in the control and reference diets, and the homogenous distribution and stability of the trait proteins in the TC1507 diets were confirmed by ELISA (data not shown). The results of nutrient composition analysis for the control, TC1507 and reference diets confirmed that they were acceptable for use in the feeding study (**Table S1 in Supplementary Information**). All the qualitative observations and numerical measurements were evaluated across endpoints for any pattern of biological effect that might be revealed despite the lack of statistical significance. The observed distribution of data across dietary groups was attributed to normal biological variation between randomly chosen samples from a population of animals. Data for animals in test groups were generally consistent with those of concurrent control and reference groups. The magnitudes of differences between groups were often minimal and without a concentration dependent relationship (i.e., the higher or lower mean values from the TC1507 High test group were not consistently of greater magnitude than those from the TC1507 Low group). No consistent patterns of behavioral or physiological dysfunction emerged across parameters (e.g., serum analytes, organ weights, and microscopic findings pertaining to a given organ system). With the exception of one male rat from the reference group XL5840, all animals survived to scheduled euthanasia. Although the cause of early death for this animal was

undetermined, it was clearly unrelated to consumption of TC1507 maize grain since this group of animals was not fed a diet containing TC1507 grain. Subchronic dietary exposure of male and female rats to TC1507 High or TC1507 Low test diets did not result in any diet-related effects on survival, clinical signs, ophthalmology, body weight (**Table S2 in Supplementary Information**) or dietary intake parameters (**Table S3 in Supplementary Information**), neurobehavioral parameters (**Tables S4-S5 in Supplementary Information**), or hematology, coagulation, clinical chemistry, or urinalysis parameters (**Tables S6-S9 in Supplementary Information**). There were no diet-related effects on organ weight parameters (**Table S10 in Supplementary Information**) nor were there any diet-related gross or microscopic observations (**Table S11 in Supplementary Information**). In fact, the solitary identified statistical difference following FDR-adjustment was a significantly lower serum AST value in the TC1507 Low group compared with the Control group. This difference was considered spurious and unrelated to consumption of the test diet based on the absence of a concentration-dependent response in the TC1507 High group, lack of differences in other parameters suggestive of a target-organ effect, and in consideration that the direction of the change is not considered toxicologically relevant.

As originally demonstrated in the previous 90-day feeding study with the TC1507 maize grain (MacKenzie et al., 2007), and substantiated in a separate 90-day feeding study with maize grain from a breeding stack containing the TC1507 event (Appenzeller et al., 2009), the present study confirmed the absence of adverse treatment-related health effects from subchronic consumption of diets containing TC1507 maize grain. These conclusions are also consistent with that of other published TC1507 maize studies (Table 2) and the prior conclusion from EFSA (EFSA, 2021a; 2021b).

Discussion

Consequences of a politicized regulatory process and highly prescriptive regulation: The consequences of the decision by the European Commission to override EFSA's expert opinion that 90-day rat feeding studies should be conducted only when other data indicate a potential hazard (hypothesis-based study design) were predominantly two-fold: 1) the development by EFSA of *de facto* regulatory requirements for hypothesis-free 90-day rat feeding studies, and 2) the requirement that studies conducted prior to 2014 (European Commission, 2013) and assessed as safe as component (single) events in breeding stacks must retroactively comply with the regulatory requirements put in place in 2013 and 2014 (European Commission, 2013)(EFSA, 2014; **Table 3 in Appendix**). Ultimately, these regulatory process changes led to a requirement by EFSA that submissions for previously approved and commercialized single component events in breeding stacks be retested in new 90-day rat feeding studies under the 2014 test design. Thus, the result was the sacrifice of

additional animals to comply with a requirement which was not initially endorsed by EFSA. This apparent dichotomy also raises the possibility of direct contradiction with the EU's Three Rs (Replacement, Reduction and Refinement) legislation which specifies that animals should be used for scientific purposes only when there is a predicted scientific benefit or educational value (European Commission, 2010).

Interestingly, a series of EU-funded studies evaluating the contribution of animal feeding trials to the overall safety assessment of GM plants was being conducted in parallel with these evolving regulations (GRACE, GTwYST and GMO90+). The conclusions from these studies, ranging in duration from 90-days to 2-years, were consistent with the original EFSA opinion that exploratory animal feeding trials do not provide information that is necessary or additive to the overall safety assessment of a GM plant, and such studies should be considered only on a case-by-case basis when there is a valid scientific hypothesis to test (Corujo et al., 2019; Coumoul et al., 2018; Steinberg et al., 2019; Zeljenková et al., 2016; Zeljenková et al., 2014). The 2013 GM legislation states that, in line with the EU legislation regarding the protection of animals used for scientific purposes (European Commission, 2010), the use of laboratory animals "should be kept to a minimum" (European Commission, 2013). Despite this, and although the legislation mandated a review of the 90-day feeding study requirement based on the outcome of these projects, these results did not lead to a change in the position of the European Commission. This lack of action is even more striking when it is considered that the pertinent scientific risk assessment bodies of 20 countries have confirmed their position that 90-day feeding studies are only required when a hypothesis leading to potential hazard has been identified (De Schrijver & Kleter, 2019). This situation with the 90-day rat feeding study, wherein animals have been sacrificed needlessly, does not exist in isolation, as multiple single-dose acute and 28-day repeated-dose rodent toxicology studies have also been required in other cases due to evolving interpretation of the regulation, as exemplified in **Box 1 in Appendix**.

Herein, we provide one example of the systematic increase in requirements in the EU for GM products, in the absence of identified hazards and driven primarily by public pressure (Brune et al., 2021; Herman et al., 2019). As described by Garcia-Alonso et al. (2022), various attempts to resolve this public debate by legal and regulatory means have created the most cumbersome and byzantine regulatory system for GM crops in the world. Although done with the best of intentions, this approach has multiple unintended side effects, including 1) misinterpretation and misrepresentation of results to the public by those who want to spread fear of new crop technologies, 2) poor use of both public and private resources, 3) misalignment between EU policies for GM crop safety evaluations and animal welfare considerations, which can impact the credibility of policy makers and regulators, 4) exclusion or delay in

commercialization of potential agricultural solutions that will ultimately contribute to the EU Commission's priorities such as the Green Deal and Farm to Fork, 5) withholding farmer access to GM crop products that have been evaluated to be as safe as conventionally bred crops, thus negatively impacts the EU's promise to create an innovation-based economy based on the principles of sustainable agriculture and food safety. With an increasing human population and the threat of climate change, it is critical that government policies encourage the development of beneficial technologies that increase sustainable agricultural production in an unbiased way (Herman et al., 2020; Herman et al., 2021; Qaim et al., 2020).

Regulatory policies and approaches could easily be modified in response to the recognition of these unintended adverse consequences, including 1) separating highly prescriptive guidance from formal regulatory requirements such that risk assessors are free to use the best available science to assess risk, 2) implementing the recommendations put forward by Garcia-Alonso et al. (2022) only sacrificing animals when scientifically necessary, 3) ensuring limited resources are not diverted to low-value studies, 4) only sacrificing animals when scientifically necessary and 5) increasing risk communication activities by involving social scientists and economists to promote a holistic thinking to allow and encourage consideration of both potential benefits and risks of alternative agricultural options without giving disproportionate weight to those ideologically opposed to modern agricultural biotechnology.

Conclusions

Time for change: The predictable innocuous results from a compulsory new 90-day rat feeding study with TC1507 maize grain are reported here to supplement the already extensive body of evidence available to policy makers indicating that consumption of TC1507 maize is as safe for humans and animals as conventional maize. Herein, we advocate that the requirement to perform 90-day rat feeding studies should be reserved for cases where other evidence from the safety assessment suggests a potential for the occurrence of an adverse effect (hypothesis-based study design). The routine conduct of animal feeding studies in support of the risk assessment for GM crops is not scientifically warranted and directly contradicts existing EU legislation regarding the protection of animals used for scientific purposes and has unintended consequences that can negatively impact progress toward EU agricultural sustainability goals.

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Appendix

Box 1: Hazard-based vs. risk-based assessment of genetically modified crops

Prescribing arbitrary high-dose requirements in regulatory oversight of toxicity testing overlooks likely exposure to the substance of interest and ignores the foundation concept in toxicology that "the dose makes the poison". Relative risk for different substances is better determined by comparing margins of exposure where an adverse effect might occur. For example, testing substances at a high dose of 100-fold likely exposure is more risk based than testing at some arbitrary high dose (e.g., 5000 mg/kg body weight). The unintended effect of setting arbitrary threshold doses in toxicity studies with newly expressed proteins in genetically modified (GM) crops is exemplified as follows.

- Acute oral toxicity studies in mice are required by most regulatory authorities for newly expressed proteins in GM crops. Such a study was conducted with the aryloxyalkanoate dioxygenase 12 (AAD-12) enzyme that degrades the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). AAD-12 is expressed in GM event DAS-444Ø6-6 soybean (at approximately 34 ng/mg in raw grain) rendering it tolerant to 2,4-D. Processing soybeans so that they are safe for consumption by humans and for monogastric animals requires conditions which denature many proteins and results in non-detectable AAD-12 protein levels in soybeans processed for human and monogastric animal consumption. An initial study demonstrated that an AAD-12 dose of 2000 mg/kg bodyweight (equivalent to 55 kg person consuming over three metric tons of raw DAS-444Ø6-6 soybean seed containing 34 ng/mg of AAD-12) resulted in no treatment-related adverse effects. However, to meet regulatory requirements in China (MOA, 2016), a second study, which also resulted in no treatment-related adverse effects, was conducted at a dose of 5000 mg/kg bodyweight (Herman et al., 2018; Papineni et al., 2017).

- To meet unique and evolving requirements in the European Union, three separate repeated-dose 28-day mouse studies were conducted with the AAD-12 protein. In the first study, mice were fed a high dose of 47 mg/kg bodyweight which is equivalent to a 55-kg human consuming 75 kg of raw DAS-444Ø6-6 soybean seed each day. Evolving interpretation of regulatory guidance by EFSA resulted in the performance of two additional 28-day mouse studies with AAD-12 protein at doses up to 1000 mg/kg bodyweight (now routinely required irrespective of conservatively estimated exposure levels) (Herman et al., 2018; Papineni et al., 2018).

Generally requiring animal toxicity studies with newly expressed proteins in GM crops is not scientifically warranted in the first place since most dietary proteins are nutrients rather than toxicants. As previously described, the characteristics of a GM trait should determine if animal studies are warranted to assure safety (Delaney et al., 2008). When animal toxicity studies are warranted to assess safety, the selection of doses should be based on multiples of estimated exposure and not set at an arbitrary value applied to all substances irrespective of exposure.

Table 1: European Union Assessments of Breeding Stacks Containing the TC1507 Event

Event	Application (Scope) Opinion	Herbicide Regime GM	Biological Relevance*
1507xNK603	EFSA-GMO-UK-2004-05 (EFSA, 2006)	+ glu	No
1507x59122	EFSA-GMO-NL-2005-15 (EFSA, 2009b)	+/- glu	No
59122x1507xNK603	EFSA-GMO-UK-2005-21 (EFSA, 2009c)	+ glu (& gly)	No
MON89034x1507xMON88017x59122	EFSA-GMO-CZ-2008-62 (EFSA, 2010b)	+ glu (& gly)	No
MON89034x1507xNK603	EFSA-GMO-NL-2009-65 (EFSA, 2010a)	+ glu (& gly)	No
Bt11x59122xMIR604x1507xGA21	EFSA-GMO-DE-2011-99 (EFSA, 2016)	+/- glu (& gly)	No (see Table 6 in EFSA opinion document)
1507x59122xMON810xNK603	EFSA-GMO-NL-2011-92 (EFSA, 2017a)	+/- glu (& gly)	No
MON87427xMON89034x1507xMON88017x59122	EFSA-GMO-BE-2013-118 (EFSA, 2017b)	+/- glu (& gly)	Thiamin was assessed
Bt11xMIR162x1507xGA21	EFSA-GMO-DE-2010-86 (EFSA, 2018b)	+ glu (& gly)	beta-carotene was assessed
MON89034×1507×NK603×DAS40278	EFSA-GMO-NL-2013-112 (EFSA, 2019b)	+/- glu (& gly, 2,4-D, AOPP)	In forage total fat; in grain cysteine, isoleucine, phenylalanine, raffinose, manganese, beta-carotene
MON89034×1507×MON88017×59122×DAS40278	EFSA-GMO-NL-2013-113 (EFSA, 2019a)	+/- glu (& gly, 2,4-D, AOPP)	Glutamic acid, glycine, leucine, lysine, threonine, protein, magnesium, manganese
Bt11×MIR162×MIR604×1507×5307×GA21	EFSA-GMO-DE-2011-103 (EFSA, 2019c)	+ glu (& gly)	Ash, Potassium, zinc, beta-carotene, folic acid, methionine, arachidic acid, ferulic acid

Herbicide regime note: + indicates the plants were treated with the specified herbicide regime; +/- indicates the study contained two entries of the GM, where one entry was treated with the specified herbicide regime and the other entry was not; glu indicates a glufosinate-containing herbicide; gly indicates glyphosate-containing herbicide; 2,4-D indicates a 2,4-dichlorophenoxyacetic acid -containing herbicide. AOPP indicates aryloxyphenoxypropionate-containing herbicide.

*Statistical differences noted in the EFSA opinion that EFSA further evaluated.

Table 2: Nutritional Equivalence/Livestock Performance Studies with TC1507 Single Event

Species	Authors	Description and Conclusion
Broiler Chicken	McNaughton and Zeph, 2004	Broiler study nutritional evaluation of b.t.cry1f maize corn from *bacillus thuringiensis* subsp. *aizawai* and phosphinothricin-n-acetyltransferase. Conclusion: Maize grain from TC1507 is considered nutritionally equivalent to maize grain from commercial lines.
Beef Heifers	Sindt et al., 2007	Effect of corn containing Cry1F protein on performance of beef heifers fed a finishing diet based on steam-flaked corn. The Professional Animal Scientist. 23(6):632-636 https://doi.org/10.15232/S1080-7446(15)31033-0 Conclusion: Growth performance and carcass characteristics were not significantly different between beef heifers fed diets with maize grain containing the event TC1507 when compared to those fed diets containing grain from the near-isoline control or reference maize.
Laying Hen	Scheideler et al., 2008	Evaluation of nutritional equivalency of corn grain from DAS-Ø15Ø7-1 (Herculex* I) in the diets of laying hens. Journal of Applied Poultry Research. 17(3):383-389. https://doi.org/10.3382/japr.2007-00080 Conclusion: Layers fed diets containing maize grain containing the event DAS-Ø15Ø7-1 performed as well as hens fed diets containing grain from the near-isoline control or reference maize.
Swine	Stein et al., 2009	Growth performance and carcass composition of pigs fed corn grain from DAS-Ø15Ø7-1 (Herculex* I) hybrids. The Professional Animal Scientist. 25(6):689-694 https://doi.org/10.15232/S1080-7446(15)30776-2 Conclusion: Performance and carcass quality of pigs fed diets containing maize grain containing the event DAS-Ø15Ø7-1 were similar to pigs fed diets containing grain from the near-isoline control or reference maize.
Dairy Cows	Faust et al., 2007	Performance of lactating dairy cows fed silage and grain from a maize hybrid with the *cry*1F trait versus its nonbiotech counterpart. Journal of Dairy Science. 90(12):5706-5713 https://doi.org/10.3168/jds.2007-0480 Conclusion: Milk production, milk composition, and cow health for dairy cows fed diets containing maize grain plus silage from TC1507 was no different from dairy cows fed the near-isoline control maize grain plus silage.

Table 3: 90d Rat Feeding Study Requirements (OECD vs. EFSA 2011, vs. EFSA 2014, vs Original 1507 Study, vs New 1507 Study)

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
Study design						
animal species	rat preferred; mouse may be used	rats preferred; mouse may be used	rats (outbred) preferred; mouse may be used	rats preferred; mouse may be used	Crl:CD (SD)IGS BR rats	Crl:CD (SD) rats
macro and micro-environment	temp: 22C +/- 3C RH: 30-70% light: 12h/12h feed: ad libitum	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)[b]	met OECD requirements	temp: 20-25C RH: 30-70% light: 12h/12h feed: ad libitum
housing	not specified	pairs unless justified	pairs recommended; individual housing should be approved by animal welfare body and justified	Small groups; individually when justified	individually housed	pair housed
allocation of animals to cages and cages to racks	random allocation to cages; minimize possible cage effects on racks	completely randomized or RCBD	same as EFSA (2011)	same as OECD 408 (1998)	random allocation; racks relocated within room and cages repositioned on racks every two weeks	randomized to cages and diet groups; blocks constructed with six pairs of each sex with the lowest mean body weight assigned to the first block, six pairs with the next lowest mean body weight to the second block and so on. For each block, pairs were randomized to a position on a cage rack, where cages of each sex in a block were grouped together
blinding	not specified	yes, except histopathology	same as EFSA (2011)	same as OECD 408 (1998)	not blinded	blinded

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
number and sex	minimum 10/sex; additional for interim or recovery sacrifice	number determined by power analysis or by SES approach	number not specified for scenario 2	same as OECD 408 (1998)	12/sex	16/sex
group numbers	three test substance dose levels one concurrent control	two test substance dose levels one control	same as EFSA (2011); Only top dose for scenario 2	same as OECD 408 (1998)	test high group test low group control high group control low group reference group	test high group test low group near-isogenic control group three reference groups
dose levels	Elicit high-dose adverse effect, graded effect at mid-dose and no effect at low-dose (ideal).	test high at max incorporation rate test low at 0.25 to 0.50 max and above anticipated human intake	same as EFSA (2011) & see crop incorporation rates		Test high: 33% Test low: 11%/22% reference Control high: 33% Control low: 11%/22% reference	Test high: 50% test Test low: 33% test/17% control Control: 50% control
control group	untreated or vehicle control	non-GM line with comparable genetic background; near-isogenic or isogenic depending on crop type	same as EFSA (2011)	same as OECD 408 (1998)	near-isogenic	near-isogenic
reference groups	not specified	not recommended; include when needed; justify	not recommended, but not excluded	same as OECD 408 (1998)	one reference (same reference used with 11% test and 11% control)	three reference groups
test substance characterization	not specified	name source composition manufacturing process stability genetic event (molecular) protein concentration	same as EFSA (2011)	same as OECD 408 (1998)	maize grain sources: name source nutrient composition contaminants event presence event absence	maize grain sources: name source nutrient composition contaminants event presence event absence protein concentration

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
crop incorporation rate	not specified	max incorporation should not induce nutritional effects	maize: 50%	same as OECD 408 (1998)	maize: 33%	maize: 50%
diet characterization	not specified	macro-nutrients micro-nutrients anti-nutrients contaminants stability homogeneity	refer to EFSA (2011): added molecular and protein characterization	not specified	macro-nutrients micro-nutrients anti-nutrients contaminants stability event presence/absence protein concentration	macro-nutrients micro-nutrients anti-nutrients contaminants protein concentration stability homogeneity event presence/absence
feeding period	min 90 consecutive days	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)	min 90 consecutive days	min 90 consecutive days
for herbicide tolerant plants, test material should be from sprayed entry	not specified	not specified	requirement	same as OECD 408 (1998)	test material was not from sprayed plants	test material was from plants sprayed with glufosinate
In-life						
mortality morbidity	2x daily	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)
general clinical observations	1x daily	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	2x daily	same as OECD 408 (1998)
body weight	weekly	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	daily for first week; weekly thereafter	same as OECD 408 (1998) and on days of neurobehavioral evaluation
feed consumption	weekly	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	daily for first week; weekly thereafter	weekly interval; by cage

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
detailed clinical observations	weekly	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998) **	same as OECD 408 (1998)
ophthalmological exam	pretest and near end of in-life	refers to OECD 408 (1998) and OECD 407 (2011)	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998)
FOB/MA	pretest (optional) near end of in-life can be waived if evaluated in other studies.	refers to OECD 408 (1998) and OECD 407 (2011)	Recommended if indicated by other study observations	eliminate when no relevant clinical observations	during acclimation and during week 13	during acclimation and during week 13
Clinical Pathology						
hematology[C]	just prior to euthanasia includes: RBC, HCT, MCH, MCHC, WBC, ANEU, ALYM, AMON, AEOS, ABAS, PLT	refers to OECD 408 (1998) and OECD 407 (2011) which also includes ARET	same as OECD 408 (1998); suggested MCV, RDW, ARET	same as OECD 408 (1998) added ARET	just prior to euthanasia; same as OECD 408 (1998) as well as sample condition, MCV, RDW, ARET, ALUC	just prior to euthanasia; same as OECD 408 (1998) as well as WB, HGB, MCV, ALUC, AIL, AIM, ARET
blood clotting time/potential[d]	required	refers to OECD 408 (1998) and OECD 407 (2011)	conducted same as OECD 408 (1998); clarified refers to PT and APTT	same as OECD 408 (1998)	PT, APTT	PT, APTT
clinical chemistry[e]	just prior to euthanasia; after overnight fast; includes Na, K, GLUC, CHOL, urea, BUN, CREA, TP, ALB, more than two of ALKP, ALT, AST, GGT, SDH; optional other enzymes and TBA	just prior to euthanasia; after overnight fast; refers to OECD 408 (1998) and OECD 407 (2011) which also includes TBA; optional other enzymes and BILI	just prior to euthanasia; after overnight fast; requires OECD 408 (1998) parameters but instead of more than two requires more than three of ALKP, ALT, AST, GGT, SDH; recommends BILI, TBA, CL, Ca, IPHS, TRIG	just prior to euthanasia; after overnight fast; Na, K, GLUC, CHOL, HDL, LDL, urea, BUN, CREA, TP, ALB, more than two of AKLP, AST, ALT, GGT, SDH; optional other enzymes and BILI, T3, T4, TSH	just prior to euthanasia; after overnight fast; Na, K, BUN, CREA, ALKP, ALT, AST, SDH, ALB, TP, GLUC, CHOL	just prior to euthanasia; after overnight fast; AST, ALT, SDH, ALKP, BILI, BUN, CREA, CHOL, TRIG, CLUC, TP, ALB, GLOB, Ca, IPHS, K, Cl, TBA, NDHL, HDLC, T3, T4, TSH

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
Urinalysis[f]	optional during last week; appearance, volume, osmolality or specific gravity, pH, protein, glucose, blood/blood cells	refers to OECD 408 (1998) and OECD 407 (2011)	refers to OECD 408 (1998); suggested creatinine	same as OECD 408 (1998)	quality, color, clarity, volume, osmolality, pH, glucose, ketone, bilirubin, blood, urobilinogen, protein, microscopic urine sediment examination	quality, color, clarity, volume, pH, specific gravity, glucose, ketone, bilirubin, blood, urobilinogen, protein as well as microscope examination of urine sediment
Anatomic Pathology						
gross necropsy	includes examination of body surface, all orifices, and the cranial, thoracic, and abdominal cavities and their contents	refers to OECD 408 (1998) and OECD 407 (2011)	full macroscopic evaluation	same as OECD 408 (1998)	same as OECD 408 (1998)	same as OECD 408 (1998) and pelvic cavities
organ weights	all animals/all groups; organ weights taken for liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart	all animals/all groups; refers to OECD 408 (1998) and OECD 407 (2011) which also includes weight of prostate + seminal vesicles with coagulating glands as a whole; OECD 407 (2011) optional weights of paired ovaries, uterus including cervix, and thyroid	all animals/all groups; refers to OECD 408 (1998) plus spleen weight	all animals/all groups: liver, kidneys, adrenals, testes, epididymides, prostate plus seminal vesicles with coagulating glands as a whole, uterus, ovaries, thymus, spleen, brain, heart, pituitary gland, thyroid	all animals/all groups	all animals/all groups: accessory sex organs, adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, pituitary gland, prostate, seminal vesicles, spleen, testes, thyroid gland, uterus
histopathology	control and high dose groups; others if - treatment related changes observed in high dose group[f]	control and high dose groups; refers to OECD 408 (1998) and OECD 407 (2011); which also includes eye, uterus **and** cervix, epididymides, prostate **plus** seminal vesicles and coagulating	control and high dose groups; refers to OECD 408 (1998), epididymides (suggested), femur, ovaries, rectum, salivary glands, sciatic nerve, skeletal muscle,	control and high dose groups; same as OECD 408 (1998); added ovaries, cervix, vagina, testes, epididymides, seminal vesicles,	test (33%) and control (33%)	control and test high; same as EFSA (2014) and/or OECD 408 (2018)

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
		glands, vagina, skeletal muscle, bone	sternum with bone marrow, testes, tongue, trachea, vagina (suggested)	coagulation glands, mammary gland (male), skeletal muscle, bone		
Statistical analysis						
methods	Statistical methods and data to be analyzed should be selected during study design	Overview of statistical methods, including design and analysis should be documented in protocol prior to start of trial; SAP should be written and signed off prior to end of experiment	refer to EFSA (2011)	same as OECD 408 (1998); for quality control, control data compared to HCD from same lab, species, strain, and collected under similar conditions	Defined in the study protocol	Defined in the study protocol
experimental unit	not specified	cage (pair of same gender)	refer to EFSA (2011)	not specified	individual animal/cage	Potential correlation between individuals within the same cage addressed using a compound symmetry variance-covariance structure in the linear mixed model
combined gender analysis	not specified	Required	refer to EFSA (2011)	not specified	males and females were analyzed separately	Combined gender analysis was conducted if a test of negligible interaction between diet and gender was non-significant
SES reporting	not specified	Required	refer to EFSA (2011)	not specified	not required	SES values were reported and graphed, but were not interpreted

Study parameters	OECD 408 (1998)	EFSA (2011)	EFSA (2014) Scenario 2[a]	OECD 408 (2018)	Original TC1507 Study (TC1507 untreated) Mackenzie et al., 2007	New TC1507 Study (TC1507 herbicide treated)
multiplicity adjustment	not specified	should be addressed in the protocol and SAP; methods clearly documented and referenced	refer to EFSA (2011)	not specified	not required	A multiplicity adjustment was applied using the FDR method of Benjamini and Hochberg (Benjamini and Hochberg, 1995; Westfall et al., 1999)
compare test to	not specified; evaluation using appropriate and generally acceptable statistical method	to isogenic control; natural variation derived from HCD (reference groups not recommended)	refer to EFSA (2011)	same as OECD 408 (1998)	test compared to isogenic control; if significant differences observed, compared to reference	for each sex and for both sexes combined (as applicable), test high compared to control and test low compared to control; evaluation based on direction and magnitude of observation, incidence and/or severity, natural range of variation and evaluation of corroborative differences in related response variables; facility HCD were utilized as needed

[a]*Scenario 2: No relevant changes and/or specific hazards were identified therefore it was not possible to identify a hypothesis (EFSA, 2011).*

[b]*New requirement was added to avoid diets of bedding with hormonally-active substances such as phytoestrogens (OECD, 2018).*

[c] *Abbreviations for hematology parameters are as follows: red blood cell count (RBC), hematocrit (HCT), mean corpuscular (cell) hemoglobin (MCH), mean corpuscular (cell) hemoglobin concentration (MCHC), white blood cell count (WBC), absolute neutrophil (ANEU), absolute lymphocyte (ALYM), absolute monocyte (AMON), absolute eosinophil (AEOS), absolute basophil (ABAS), platelet count (PLT), absolute reticulocyte (ARET), mean corpuscular (cell) volume (MCV), red cell distribution width (RDW), whole blood condition (WB), hemoglobin (HGB), absolute large unstained cell (ALUC), absolute immature lymphocyte (AIL), absolute immature, and monocyte (AIM).*

[d]*Abbreviations for coagulation parameters are as follows: plasma hemolysis (PHEM), plasma lipemia (PLIP), plasma icterus (PICT), prothrombin time (PT), and activated partial thromboplastin time (APTT).*

[e]*Abbreviations for clinical chemistry are as follows: sodium (Na), potassium (K), glucose (GLUC), cholesterol (CHOL), urea nitrogen (BUN), creatinine (CREA), total protein (TP), albumin (ALB), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), sorbitol dehydrogenase (SDH), total bile acids (TBA), total bilirubin (BILI), chloride (CL), calcium (Ca), inorganic phosphorus (IPHS), triglycerides (TRIG), high-density lipoprotein (HDL), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein (LDL), non-high-density lipoprotein cholesterol (HNDL), hemolysis (HEM), lipemia (LIP), icterus (ICT), globulin (GLOB), triiodothyronine (T3), thyroxine (T4), and thyroid stimulating hormone (TSH).*

[f]*Histopathology should include the following: gross lesions, brain (including cerebrum, cerebellum, medulla/pons), spinal cord (cervical, mid-thoracic, lumbar), pituitary, thyroid, parathyroid, thymus, esophagus, salivary glands, stomach, small and large intestines including Peyer's patches, liver, pancreas, kidneys, adrenals, spleen, heart, trachea, lungs, aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, lymph nodes, peripheral nerve, bone marrow (section), skin, eyes (if needed).*