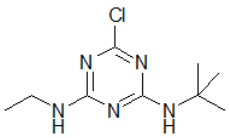
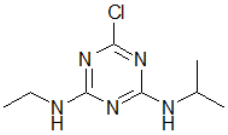
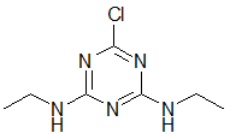
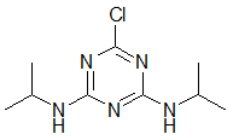
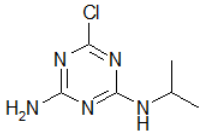
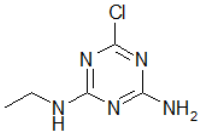
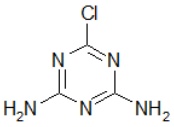
TERBUTHYLAZINE – POSITION ON MAMMARY TUMOURS IN RATS

In a chronic toxicity and carcinogenicity study with Sprague Dawley-derived rats (Gfeller, 1983), an increased incidence of mammary gland adenocarcinoma was noted for female rats receiving the highest dose of terbuthylazine tested (750 ppm).

The mode of action for induction of mammary tumours in Sprague Dawley, but not Fischer 344, rats by chlorotriazine herbicides is well understood and is considered not relevant to humans (summarised in Simpkins *et al*., 2011). The majority of the relevant mode of action investigations have been conducted using the class exemplar atrazine, with the outcomes extrapolated to the other chlorotriazines (and selected metabolites) owing to a shared herbicidal mode of action and very high degree of structural similarity (see Table 1).

Table 1. Chlorotriazine herbicides (and metabolites) share a high degree of structural similarity

Chlorotriazine	Terbuthylazine	Atrazine	Simazine	Propazine
Structure				
Metabolite	**DEA**	**DIA**	**DACT**	
Structure				

Owing to the shared herbicidal mode of action, similar toxicology databases and very high degree of structural similarity, the United States Environmental Protection Agency (US-EPA, 2002, 2006 and referenced in EFSA 2103) has evaluated this class of chemistry and determined that atrazine, simazine, propazine, DEA, DIA and DACT:

"*will be considered as a common mechanism group for purposes of a cumulative risk assessment and as part of the tolerance reassessment process for triazine pesticides*" (US-EPA, 2002).

Terbuthylazine was not considered in this assessment because it is not registered in the US. However, based on structural similarity, as well as a similar toxicological database, terbuthylazine can be reasonably considered part of this common mechanism group.

A second chronic toxicity and carcinogenicity study was conducted with terbuthylazine, using Han Wistar rats (Ramesh, 2001). An increased incidence of mammary gland adenocarcinoma was noted for female rats receiving the highest dose of terbuthylazine tested (120 ppm).

Although less data regarding the mode of action for induction of mammary gland adenocarcinomas by chlorotriazines in Han Wistar rats, sufficient data are available to support Syngenta/Oxon's position that the mode of action demonstrated for Sprague Dawley rats can be reasonably extrapolated to Han Wistar rats because:

1. **Atrazine and terbuthylazine both suppress the luteinizing hormone (LH) surge in both Sprague Dawley and Han Wistar rats, and the levels of suppression are comparable.** Suppression of the LH surge (via effects on gonadotropin releasing hormone [GnRH] release) is a crucial key event for the induction of mammary tumours in Sprague Dawley rats by chlorotriazines, with a lack of an effect on this parameter being the reason why chlorotriazines do not induce mammary tumours in Fischer 344 rats (Simpkins *et al*., 2011). Mode of action work with the class exemplar atrazine has demonstrated that it can inhibit the LH surge in both Sprague Dawley (Simpkins *et al*., 2011) and Han Wistar (Foradori *et al*., 2009) rats, but not in Fischer 344 rats (Simpkins *et al*., 2011). Furthermore, recently completed mode of action studies have demonstrated that both atrazine and terbuthylazine suppress the LH surge in both Sprague Dawley and Han Wistar rats and that the levels of suppression are comparable when the two chlorotriazines are dosed in equimolar quantities (100 mg/kg/day and 106.5 mg/kg/day for atrazine and terbuthylazine, respectively) (Handa, 2014; Stump, 2014). A summary of these data are presented in Figure 1.

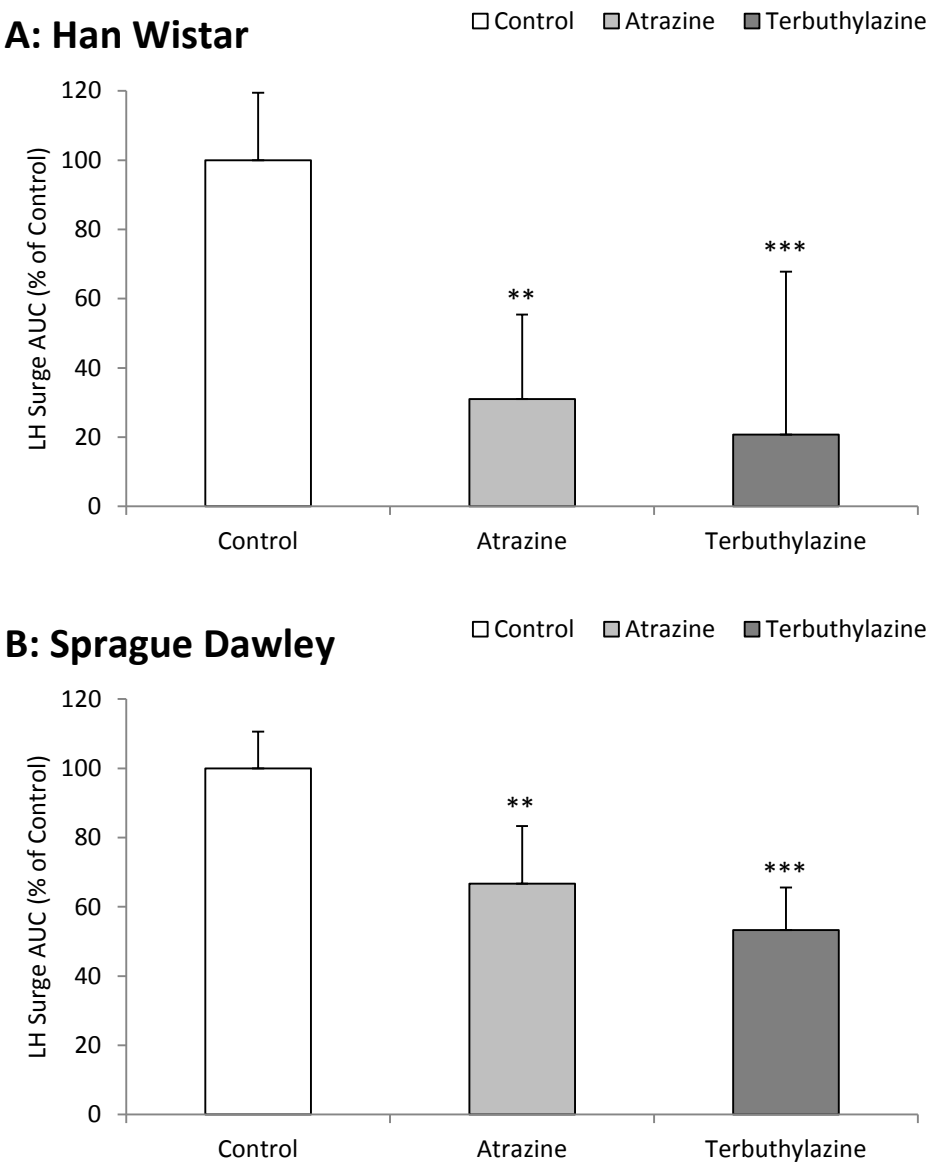


Figure 1. Atrazine and terbuthylazine both suppress the LH surge in both A: Han Wistar (from Handa, 2014) and B: Sprague Dawley (from Stump, 2014) rats. Atrazine and terbuthylazine were dosed at molar equivalent doses (100 and 106.5 mg/kg/day for atrazine and terbuthylazine, respectively). Data are area under the curve (AUC) values presented as % of Control. ** and ***: statistically significantly different from Control with $p<0.01$ and $p<0.001$, respectively. No statistically significant differences were observed between atrazine and terbuthylazine treated groups.

2. **The reproductive ageing processes for female Sprague Dawley and Wistar rats are very similar and under similar neuroendocrine control.** And therefore would be expected to respond similarly to suppression of the LH surge. The similarities between the strains are likely a result of their shared ancestry, with Sprague Dawley rats originally derived from Han Wistar rats (White and Lee, 1988). In contrast, the Fischer 344 rat is an inbred strain of non-related origin.

    a. **The primary reason for onset of reproductive senescence is the same for female Sprague Dawley and Wistar Rats.** The primary reason for onset of reproductive senescence in both of these strains is hypothalamic failure to stimulate LH (and follicle stimulating hormone [FSH]). Chlorotriazine-mediated LH suppression therefore mimics the primary reason for onset of senescence in these strains and accelerates the process, leading to earlier onset. This is in contrast to Fischer 344 rats, where the primary reason for onset is a hypothalamic failure to control prolactin surges (US-EPA, 2000a).

    b. **Both Sprague Dawley and Han Wistar rats primarily enter into a state of constant oestrus following onset of reproductive senescence.** Therefore, earlier onset of senescence in these strains will result in greater oestrogenic exposure, which is a causal key contributing to the development of mammary tumours (Simpkins *et al*., 2011). This is in contrast to Fischer 344 rats, which primarily enter into a state of pseudopregnancy/persistent dioestrus following onset of reproductive senescence, which is associated with higher progesterone exposure (US-EPA, 2000a).

    These similarities/differences between different strains of rat have been summarised by US-EPA as part of a Scientific Advisory Panel (SAP) meeting for Atrazine Cancer Risk Assessment (US-EPA, 2000a). The relevant schematic is copied below as Table 2.

Table 2. Summary of the reproductive ageing process in different rat strains.
Taken directly from US-EPA, 2000a (Part B)

Sprague-Dawley, Long Evans, Wistar[1]
▸ Normal cycle is a four to five day cycle with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
▸ Reproductive aging becomes evident at approximately nine to 12 months;
▸ Reproductive aging is characterized by decreased gonadotropin surges that leads to maintenance of primary, secondary and antral ovarian follicles;
▸ An irregular cycling pattern develops followed by an increase in the days spent in estrus, and prolonged exposure to estrogen;
▸ Pituitary alterations such as increase in pituitary weight, increases in pituitary hyperplasia and pituitary -adenomas become common as the animal ages;
▸ Acyclicity develops in the final months of life
▸ Normal cycling ➜ irregular cycles ➜ prolonged estrus ➜ acyclicity *(begins around 5-6 weeks old)* *(occurs in the last few months of life ≥ 21 months of age)*
[1]There may be temporal differences between and among strains

F-344
▸ Normal cycle is a four to five days with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
▸ Reproductive aging becomes evident at approximately 12 months;
▸ Reproductive aging is characterized by increased prolactin surges that leads to maintenance of the corpea lutea;
▸ There is an increase in the days spent in diestrus, and increased exposure to progesterone.
▸ In very aged animals, acyclicity is common

As can be seen from Table 2 above, female rat reproductive ageing in the Han Wistar and Long Evans strains is considered to be similar to the Sprague Dawley.

In US-EPA, 2000a (Part B), the following statement is made:

"*Some rat strains (LE, Wistar and SD included) undergo a similar reproductive aging process which is characterized by the appearance of persistent (or constant) estrus by approximately one year of age and under similar neuroendocrine events. Thus, the LE female rat is considered to be a valid model for evaluating atrazine's mode of action resulting in mammary tumors in SD females*"

Furthermore, in US-EPA (2000b), the following statement is made:

"*Biological plausibility has been established for the mode of carcinogenic activity of atrazine. The rat cancer mode of action (MOA) involves a process consisting of modulation of the gonadotropin releasing hormone (GnRH) pulse, attenuation of pituitary releases of luteinizing hormone (LH), and alteration of ovulatory cycles, expressed as constant estrus, which leads to prolonged exposure of mammary and pituitary tissues to estrogen and prolactin, and development of tumors in response to the prolonged hormone exposures. This MOA essentially accelerates the normal aging process in female SD rats. It would be expected to be operative in other rat strains with a similar reproductive aging process (e.g. Long Evans and Wistar)*"

These statements clearly supports Syngenta/Oxon's position that for strains of rat that undergo similar reproductive ageing processes under similar neuroendocrine control (i.e., Sprague Dawley and Han Wistar [and Long Evans]) it is appropriate to extrapolate the mode of action for chlorotriazine-induced mammary tumours in Sprague Dawley rats to the other strains.

c. **Sprague Dawley and Han Wistar rats have a very similar incidence of spontaneous mammary tumours.** This is a reflection of the fact that reproductive ageing is very similar in both strains. In contrast, control Fischer 344 rats have a lower spontaneous incidence. This is summarised in Table 3.

Table 3. Spontaneous mammary tumour incidence in various rodent strains. Adapted from US-EPA, 2000a (Part B)

Strain	Spontaneous Mammary Tumour Incidence
Sprague Dawley	~30% Fibroadenoma ~12% Carcinoma
Han Wistar	~25% Fibroadenoma ~13% Carcinoma
Fischer 344	~12% Fibroadenoma ~2% Carcinoma

d. **Prolactin is a key factor is reproductive ageing in both Sprague Dawley and Han Wistar rats.** Prolactin is key factor in chlorotriazine-induced mammary tumours (Simpkins *et al*., 2011). In contrast, prolactin is less important in Fischer 344 rats (Simpkins *et al*., 2011; Harleman *et al*., 2012).

In summary, it can be concluded that the well understood, and not relevant to humans, mode of action for chlorotriazine-induced mammary tumours in female Sprague Dawley rats can be extrapolated to Han Wistar rats, based on their shared sensitivity to chlorotriazine-mediated suppression of the LH surge and the overwhelming similarities in their reproductive ageing processes. This is in contrast to the Fischer 344 rat, which ages differently and is not sensitive to chlorotriazine-mediated suppression of the LH surge. These similarities and differences are summarised in Table 4 and overlaid onto the mode of action for chlorotriazine-induced mammary tumours in Figure 2.

Table 4. Summary of rat strain similarities/differences in sensitivity to chlorotriazine-mediated inhibition of the LH surge, reproductive ageing and spontaneous mammary tumour incidence in female rats. Adapted/compiled from Chapin *et al*., 1996; US-EPA, 2000a, 2002, 2006; Simpkins *et al*., 2011 and Harleman *et al*., 2012 [and references therein]).

	Sprague Dawley	**Han Wistar**	**Fischer 344**
Sensitivity to Chlorotriazine-Mediated Inhibition of the LH Surge	Yes	Yes	No
Age at which Reproductive Senescence Becomes Evident	~12 months	~12 months	~9-12 months
Principle Cause for Onset of Senescence	Hypothalamic failure to stimulate LH/FSH	Hypothalamic failure to stimulate LH/FSH	Hypothalamic failure to control prolactin surges
LH Surge Capability	Lost	Lost	Maintained
Predominant Cycle Pattern Post-Onset	Persistent oestrus	Persistent oestrus	Repetitive pseudopregnancy/ dioestrus
Oestrogen/Progesterone Ratio	Elevated/prolonged	Elevated/prolonged	Reduced
Prolactin Dependence	High	High	Medium
Spontaneous Mammary Tumour Incidence	~30% Fibroadenoma ~12% Carcinoma	~25% Fibroadenoma ~13% Carcinoma	~12% Fibroadenoma ~2% Carcinoma

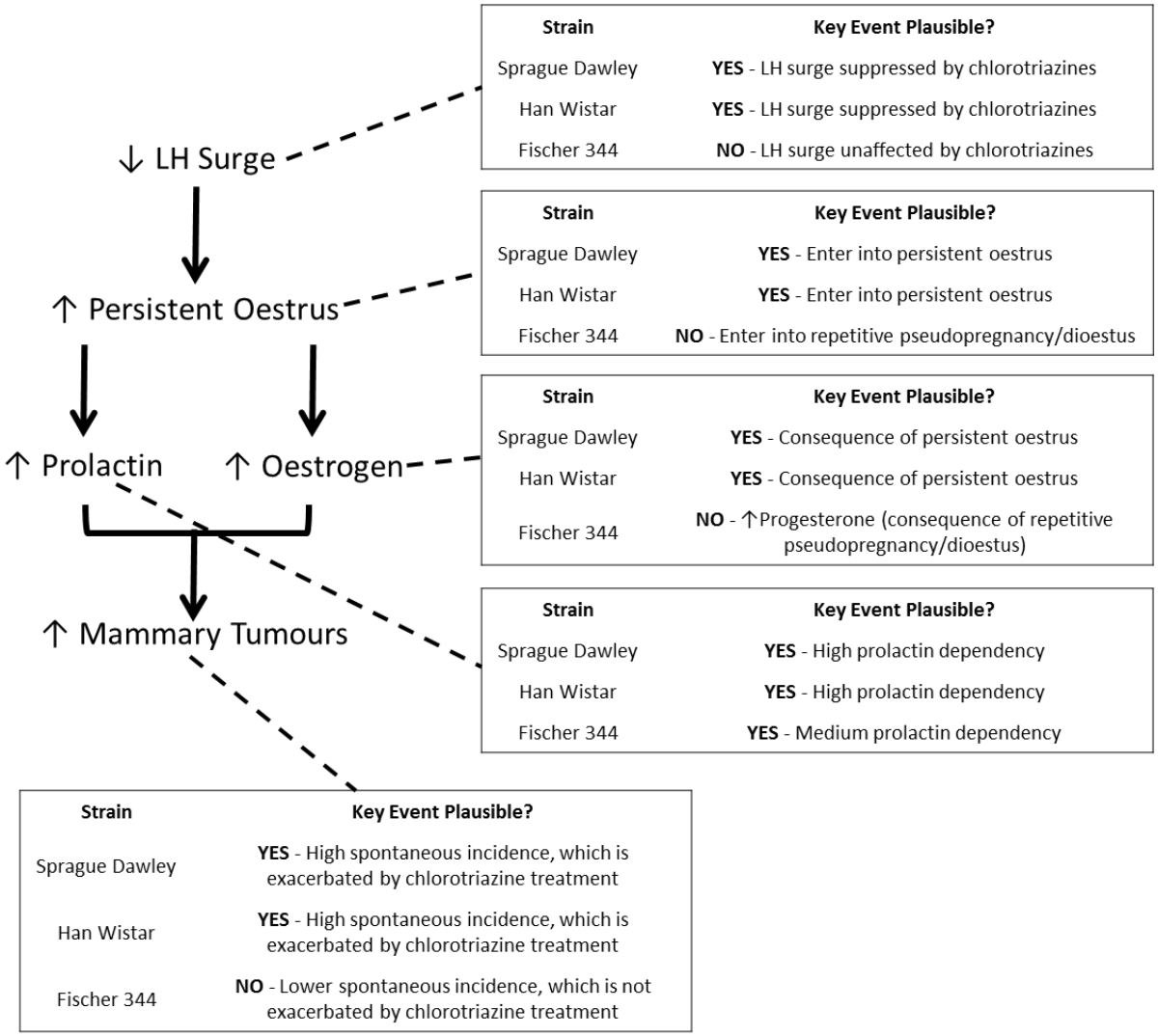


Figure 2. Mode of action for chlorotriazine-induced mammary tumours in female rats: Sensitivity of different strains. Mode of action schematic adapted from Simpkins *et al*., 2011

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