GENOTOXICITY EVALUATION OF ZAERALENONE WITH THE WING SPOT TEST IN *Drosophila melanogaster*

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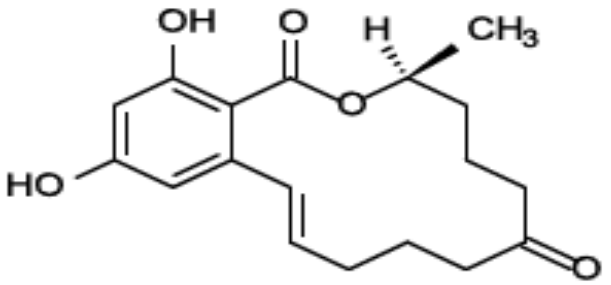
ZEARALENONE

- ZEA is a mycoestrogen produced as a secondary metabolite of *Fusarium* spp., that contaminates cereal crops worldwide, and produces mycotoxicosis of farm animals.



http://www.ncbi.nlm.nih.gov/corecgi/tileshop/tileshop.fcgi?p=PMC3&id=745010&s=34&r=4&c=5

Nesic et al. Rev Environ Contam Toxicol. 2014;228:101-20. Zhang et al. Plant Cell. Dec 2012; 24(12): 5159–5176.

ZEA HUMAN CONSUMPTION PER DAY

- The maximum tolerated levels of ZEA for human consumption have been established as:

1. 20 µg/kg bw in food intended for babies and infants.
2. 50 µg/kg bw in maize-based snacks and breakfast cereals.
3. 200 µg/kg bw in unprocessed maize and certain maize products.

Hueza et al. Toxins 2014, 6, 1080-1095.

SOME REGIONAL DIETS HAVE MORE DIETARY INTAKE (WHO)

μg/day based on 60 kg of bw

LATIN AMERICA

- Maize < 0.51
- Rice < 0.64
- Wholemeal bread < 0.42

AFRICAN

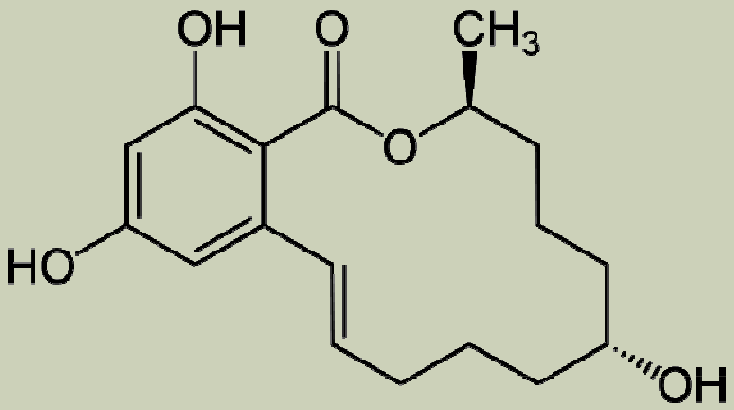
- Maize < 1.3
- Rice < 0.76
- Wholemeal bread < 0.053

A GROWTH PROMOTER FOR LIVESTOCK

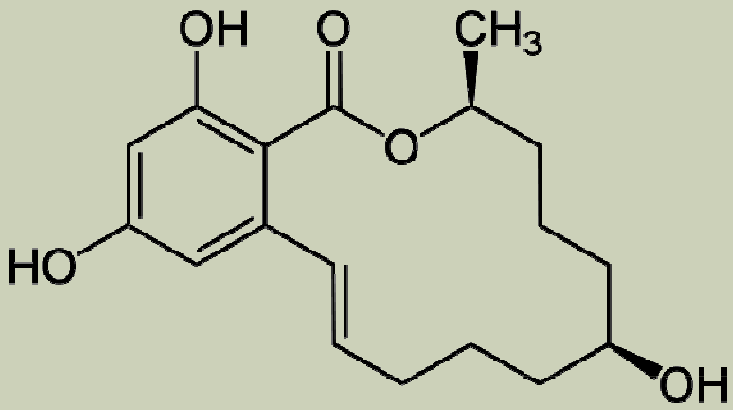
- A synthetic commercial formulation called zeranol (Ralgro®) has been marketed successfully for use as an anabolic agent for both sheep (12 mg/3 months) and cattle (36 mg/3 months).
- In 1989, zeranol (α-zearalanol) was banned by the European Union, but it is still used in other parts of the world.
- In Mexico it has official registration by the authorities (Reg. SAGARPA. Q-0552-084).

Bennett & Klich. Clin. Microbiol. Rev. 2003, 16, 497–516. Hueza et al. Toxins 2014, 6, 1080-1095.
http://www.msd-salud-animal.mx/productos/ROJO/RALGRO_/020_Informaci_n_del_producto.aspx

METABOLITES



α-ZOL



β-ZOL

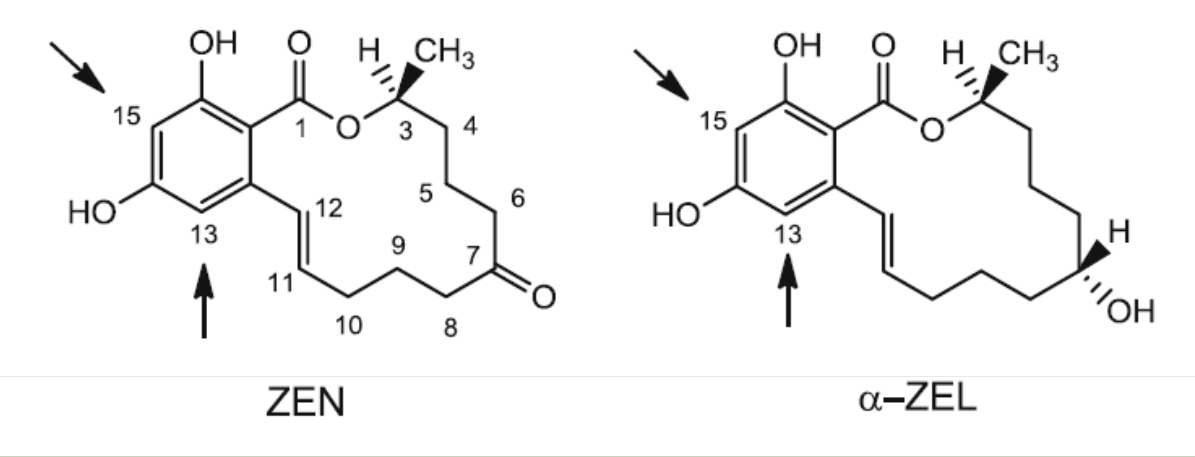
ZEA is reduced by steroid dehydrogenase metabolism to the major metabolites alpha- and beta-zearalenol (α-, β-ZOL), that have in vertebrates a higher estrogenic activity than ZEA.

SOME ZEA EFFECTS

- ZEA resembles 17β-estradiol and binds to estrogen receptors in vertebrate target cells causing hyperestrogenic syndromes.
- In mammals it have been related with an increased risk for endometrial adenocarcinomas or hyperplasia and breast cancer.
- ZEA has been associated also with oxidative damage and induction of SOS repair.

Fleck et al. Mycotoxin Res. 2012 Nov;28(4):267-73. Sáenz de Rodríguez *et al*., 1985; Tomaszewski *et al*., 1998; Abid-Essefi, *et al*., 2004; Ghedira-Chékir, *et al*., 1998.

HYDROXYLATION



As 17β-estradiol, ZEA and metabolites undergo P450s hydroxylation at the positions marked with arrows that leads to catechol metabolites or glucuronides and sulfates conjugates.

Resulting catechols can cause genotoxic effects (ROS) and may contribute to an increased cancer risk in estrogen target tissues.

Fleck et al. Mycotoxin Res (2012) 28: 267-273.

IN VITRO EFFECTS OF ZEA

- Ayed-Boussema *et al.* (2007) monitored cytotoxicity, cell cycle perturbation, genotoxicity and mutagenicity in Vero cells (kidney epithelial) exposed to ZEA.
  a) ZEA (1 -60 μM) reduced cell viability correlated to DNA fragmentation (DNA-laddering patterns).
  b) The induction of apoptotic bodies and cell cycle perturbation was demonstrated in a dose-dependent manner (ZEA 10, 20, 40 μM) .
  c) ZEA (5, 10, 20 μM) produced micronuclei and chromosome aberrations.

Ayed-Boussema *et al*. J Biochem Mol Toxicol. 2007;21(3):136-144.

GENOTOXICITY OF ZEA , ALPHA- AND BETA-ZOL

- Ayed *et al*. (2011) demonstrated in mouse bone-marrow cells* and in HeLa cells♦ that ZEA, α- and β-ZOL inhibited cell viability in a dose-dependent manner.
- ZEA and its two metabolites increased the percentage of chromosome aberrations. ZEA and α-ZOL were more genotoxic than β-ZOL.
- Authors conclude that biotransformation of ZEA may be considered as only a partial detoxification pathway since the resulting metabolites remain relatively toxic.

Ayed et al. Mutat Res. 2011 Nov 27;726(1):42-46. *100 µL of 5, 10, 20 mg/Kg; ♦ 12.5, 25 & 50% loss of cell viability.

ZEA & P450S *IN VITRO*

- Metabolic monohydroxylation of ZEA by human cytochrome P450s (CYP2C8; CYP3A4, CYP3A5) and phenobarbital-treated rat (CYP2C11) was demonstrated *in vitro* with liver microsomes yielding several OH-ZEA products limited, and not as significant on estrogen receptors as those of ZEA and α-ZOL.*
- CYP1A2 is highly active in the hydroxylation of ZEA.**

*Ding *et al.*_Toxicol Sci. 2006 Jun;91(2):448-55. Bravin *et al*. Int J Mol Sci. 2009 Apr 21;10(4):1824-37. **Drzymala *et al*. Chem Res Toxicol 2014.

THE QUESTIONS

- Is genotoxicity of ZEA caused by:
  1. Stimulation of estrogen receptors?
  2. The α- and β-ZOL metabolites?
  3. The OH-ZEA P450s products?

In an animal model without estrogen receptors and different P450s levels (induced and high) we could address questions 2 and 3.

ERR, SCU & CYP1A2

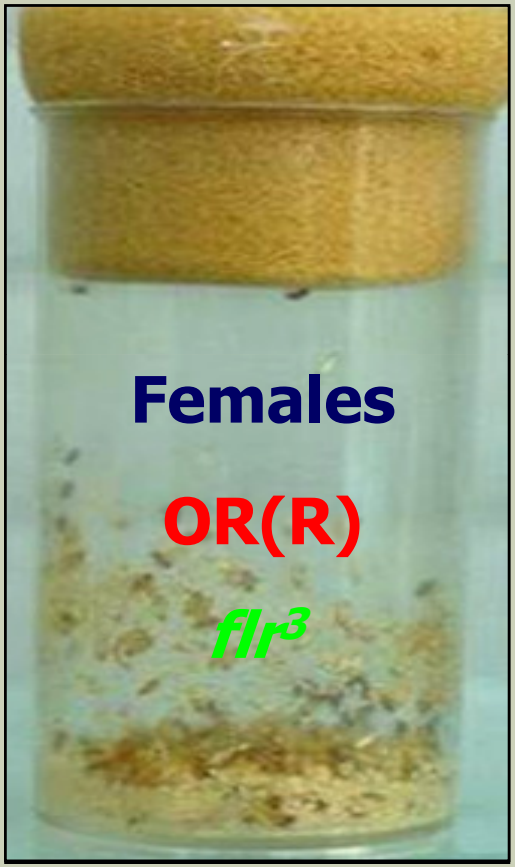
- ***Drosophila melanogaster*** has a single estrogen-related receptor *ERR* gene that codes for the dERR protein, which belongs to the orphan receptors family, with no known naturally occurring ligand, but is an essential regulator of carbohydrate metabolism during larval stages.
- It has a steroid dehydrogenase activity, acting on the CH-CH group of donors, by a protein product of the *scu* gene.
- The *Cyp1a2* gene of *Drosophila* is homolog to the human *CYP1A2* gene.

METHODS

SMART

- We evaluated the genotoxicity of ZEA (0, 100, 200, 400 μM), dissolved in phosphate buffer solution (PBS) pH 7, under and above $LC_{<25}$, with the standard (ST) and the high bioactivation (HB) crosses, with basal and high levels of P450s, respectively.

ST & HB CROSSES



Males
mwh

ST & HB Crosses

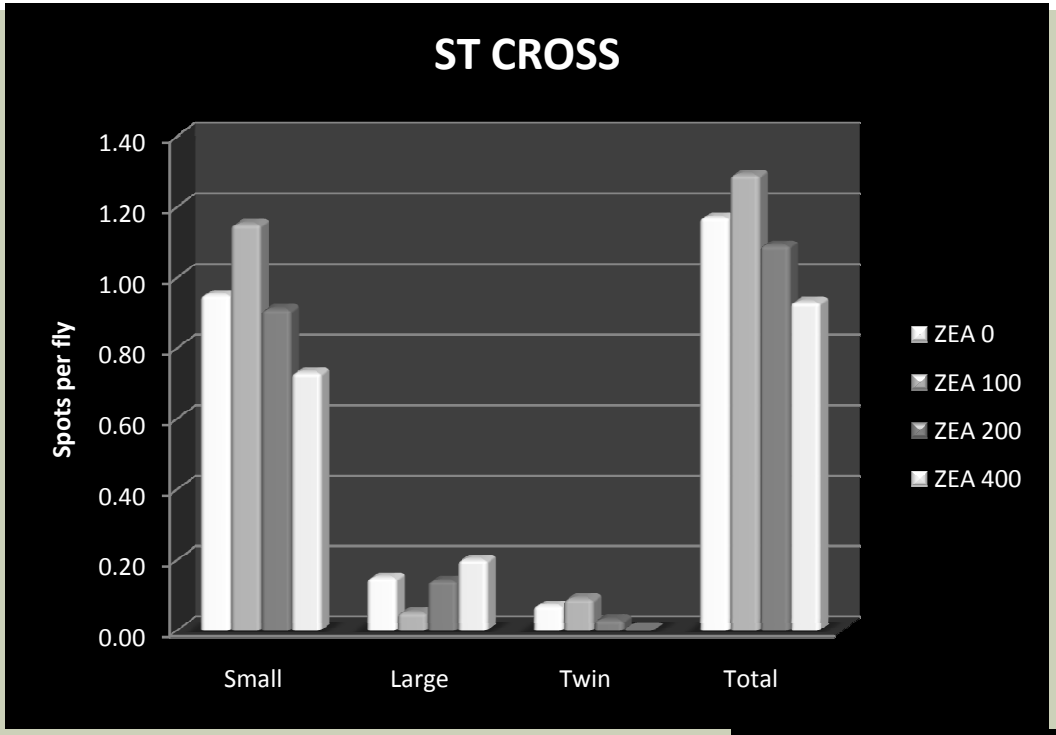
- ST (*flare X mwh*)
- HB (*Oregon-flare X mwh*)

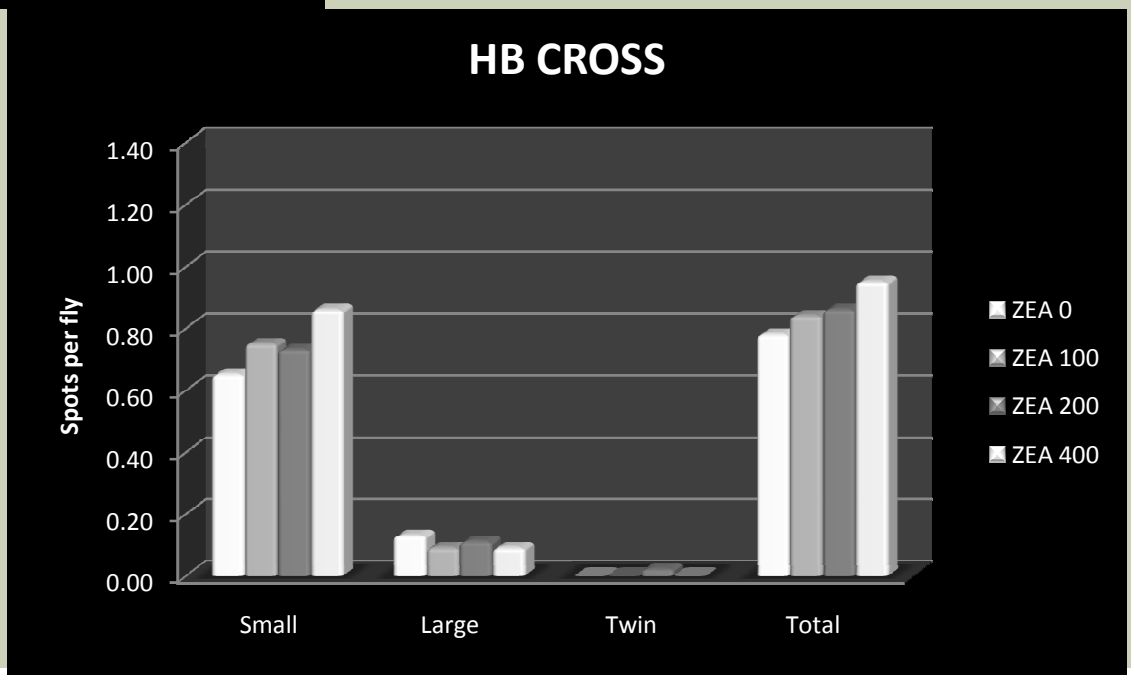
ZEA CHRONIC TREATMENTS

TREATMENT	MilliQ WATER	PO_4 BUFFER (PBS) pH 7	ZEA µM	URE mM
MilliQ water	+			
PBS		+		
ZEA		+	100	
ZEA		+	200	
ZEA		+	400	
Urethane	+			20

SMART RESULTS

ST CROSS, $p<0,05$	Number of flies	Small (1-2 cells)	Large (> 2 cells)	Twin	Total
MilliQ Water	71	0,70	0,06	0.08	0.85
PBS	59	0.95	0,15	0.07	1,17
ZEA 100 µM	55	1,15 -	0,05 -	0,09 -	1,29 -
ZEA 200 µM	58	0,91 -	0,14 -	0,03 -	1,09 -
ZEA 400 µM	55	0,73 -	0,20 -	0,00 -	0,93 -
URE 20 mM	38	3,13 ↑	0,24 ↑	0,05 ns	3,42 ↑
HB CROSS $p<0,05$	Number of flies	Small (1-2 cells)	Large (> 2 cells)	Twin	Total
MilliQ Water	60	0,75	0,10	0,02	0,87
PBS	55	0,65	0,13	0,00	0,78
ZEA 100 µM	55	0,75 -	0,09 -	0,00 -	0,84 -
ZEA 200 µM	56	0,73 -	0,11 -	0,02 -	0,86 -
ZEA 400 µM	56	0,86 -	0,09 -	0,00 -	0,95 -
URE 20 mM	39	6,31 ↑	0,62 ↑	0,26 ↑	7,18 ↑↑

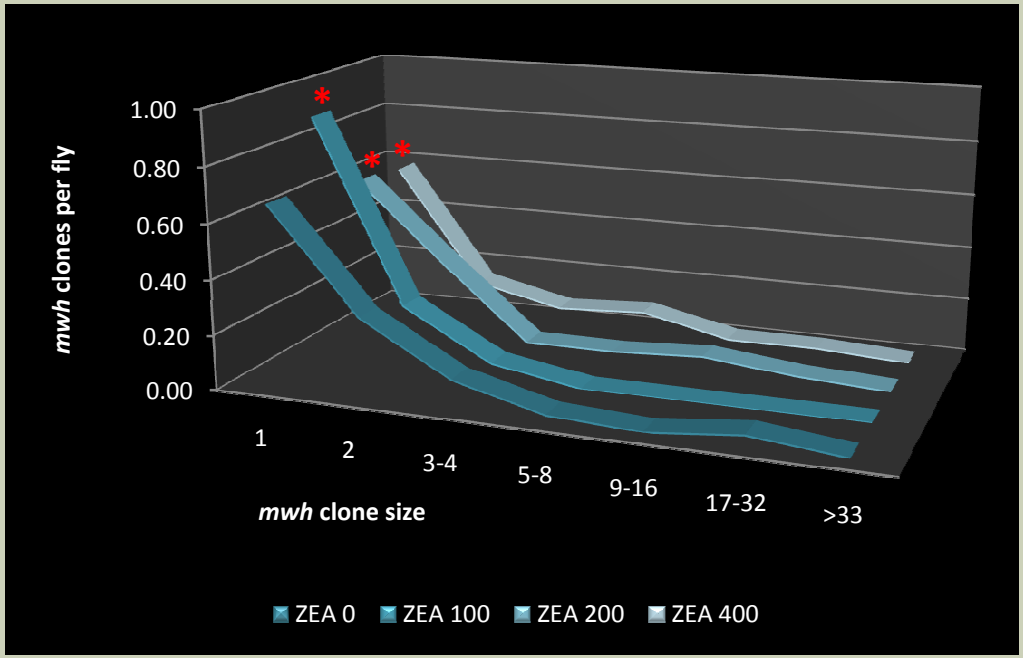

ST CROSS
Spots per fly
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Small
Large
Twin
Total
ZEA 0
ZEA 100
ZEA 200
ZEA 400


HB CROSS
Spots per fly
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Small
Large
Twin
Total
ZEA 0
ZEA 100
ZEA 200
ZEA 400

HB VS ST

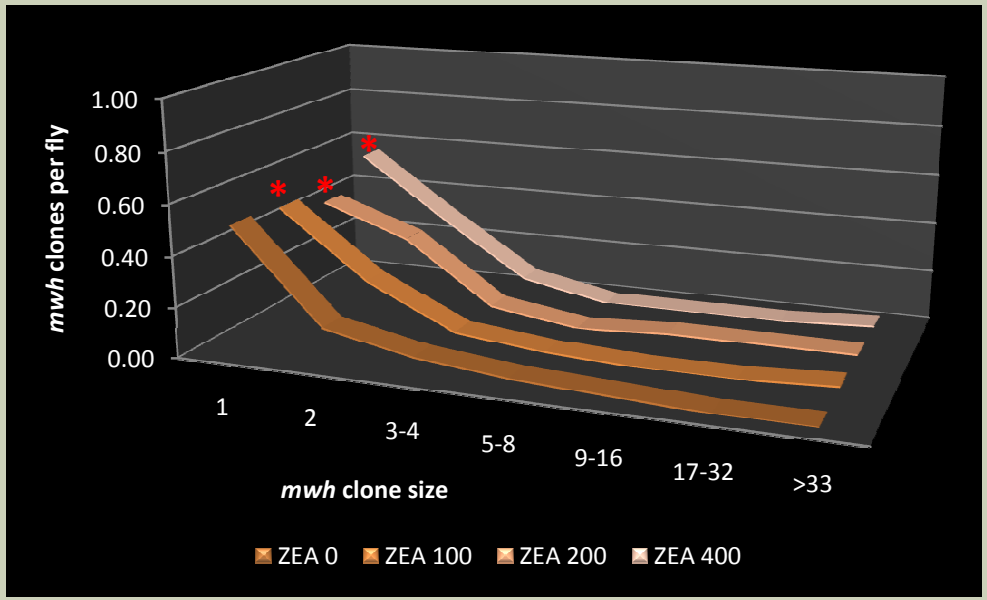
Statistical diagnoses according to Frei and Würgler (1988, 1995), $p<0.05$.	
NEGATIVE CONTROLS	**TOTAL SPOTS/FLY**
Distilled Water ST	0,85
Distilled Water HB	0,87 -
PBS ST	1,17
PBS HB	0,78 -
ZEA TREATMENTS	
100 µM ST	1,29 ↑
100 µM HB	0,84
200 µM ST	1,09 -
200 µM HB	0,86
400 µM ST	0,93 -
400 µM HB	0,95
URE POSITIVE CONTROL	
20 mM ST	3,42
20 mM HB	7,18 ↑

Distribution of *mwh* clone size



ST CROSS

HB CROSS



Clones size are produced by a number of cell division cycles after damage.

We did Kolmogorov-Smirnov analysis of distribution of the mean accumulated *mwh* clone size classes , $p < 0.05$.

DISCUSION

We have demonstrated that the *hsp60* gene is overexpressed in the flare strain (inducible P450s) treated with ZEA 260 μM (unpublished data).

Because the Hsp60 family of chaperonins has anti and pro-apoptotic properties*, we propose that in the ST cross (inducible P450s) the significant differences between small, twin and total spot frequencies from 100 vs. 400 μM, could be related to ZEA apoptotic properties.

*Arya *et al*. J Biosci 2007. 32:595–610

DISCUSION

Toxicity (unpublished data)

- ✓ In LC_{50} experiments, ZEA was not toxic in the flare or Oregon-flare strains, but the flare strain was more sensitive (LC_{20} = 155.25 μM) than the Oregon-flare strain (LC_{20} = 260 μM).

These data can explain the low total spot frequency (0.84) of the HB cross fed on ZEA 100 μM, since it presents high P450s levels, and the higher frequency in the ST cross (1.29) with inducible levels.

DISCUSION

- In the HB cross we did not find any significant differences between treatments even greater effect could be expected because the possible formation of more ZEA/α-ZOL catechol metabolites & oxidative damage.

- We propose these results can be explained by a higher detoxifying metabolism due to the high P450s levels in this cross.

CONCLUSIONS

- In contrast to previous genotoxicity reports in mammalian cells, we did not find any positive results in the *Drosophila* wing spot test, ST and HB crosses.
- Our results indicate that the ST cross is more sensitive to the tested ZEA treatments, than the HB cross. This could be explained by the difference between their CYPs levels.
- Because of the lack of an estrogen receptor in *Drosophila* results maybe due only to the effects of ZEA metabolites: alfa, beta-ZOL and OH-ZEA.

CONCLUSIONS

- It is recommended to use apoptosis markers in order to demonstrate in both crosses if this event is induced in the ST cross fed with ZEA.

- Further studies are needed to asses levels of ZEA metabolites in third instar larvae to imagos emergence of *Drosophila* flare and Oregon-flare mutant strains.

THANK YOU