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**Fate of the fusarium mycotoxins deoxynivalenol, nivalenol and zearalenone during extrusion of wholemeal wheat grain**

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**Abstract**

Deoxynivalenol in cereals and cereal products is controlled in the European Union by recent legislation with the objective of minimizing consumer exposure to this mycotoxin. Relatively few studies have examined the losses of *Fusarium* mycotoxins during processing and whether this is accurately reflected by the processing factors that have been used. The behaviour of deoxynivalenol, nivalenol and zearalenone during extrusion of naturally contaminated wholemeal wheat flour has been examined using pilot scale equipment. Factors examined were temperature and moisture content. Concentrations of the three mycotoxins were little changed by extrusion although the amount of deoxynivalenol decreased at the lowest moisture content. However, this effect did not appear to be temperature dependent and it is suggested that the apparent loss is either due to binding or inability to extract the residue. Under some conditions, concentrations of the mycotoxins, particularly nivalenol, were higher after extrusion.

**Keywords:** Deoxynivalenol, nivalenol, zearalenone, *Fusarium* mycotoxins, wheat, flour, processing, extrusion

## Introduction

Cereals worldwide are at risk from the formation of mycotoxins both in the field as the result of infection by fusarium and other fungi or after harvest because of ineffective drying or poor storage conditions. Wheat in the field is particularly susceptible to *Fusarium* head blight with the likely formation of deoxynivalenol (DON), other related trichothecene mycotoxins and zearalenone (ZON). The European Commission (EC) has recently introduced maximum permissible limits for deoxynivalenol (DON) of 1250 µg/kg in raw wheat, 750 µg/kg in flour and 500 µg/kg in finished products and is currently considering legislation for other fusarium mycotoxins in order to reduce the risk to the consumer (EC, 2006).

Extrusion processing is used for producing a range of cereal products such as breakfast foods, snacks and animal feedstuffs. Commodities such as wholemeal flour, maize flour and grits are passed through the extruder under pressure, mechanical shearing stresses and elevated temperature, and expand rapidly as they are forced through the outlet die (Riaz 2000, Guy 2001). During this process, mechanical energy is transformed into heat by viscous dissipation in the starch paste. This causes chemical bonds in the polymers to break and free radicals to form. Therefore, a contaminant such as DON is subjected both to high temperatures and to chemical reactions by free radical mechanisms and might be susceptible to some breakdown. Temperatures inside the barrel of an extruder can be as high as 200°C.

Extrusion processing has been shown to have variable effects on mycotoxins. For example, approximately 85% of aflatoxins B<sub>1</sub> and B<sub>2</sub> were lost during extrusion at 150°C when spiked corn dough was treated (Martinez and Monsalve 1989), although in a study by Cazzaniga et al. (2001), in which the effects of flour moisture content, temperature and the addition of sodium metabisulphite were examined, the reduction of aflatoxin B<sub>1</sub> was only between 10 and 25%. Boudra et al. (1995) studied the decomposition of ochratoxin A under different moisture content and temperature conditions. Their results showed that ochratoxin A could be partially decomposed. In the presence of 50% water, loss is increased at 100 and 150°C in comparison with drier grain, but is decreased at 200°C. Some loss of ochratoxin A has been found in pilot scale extrusion, although loss below 150°C was relatively small (Scudamore et al. 2004). Saunders et al. (2001) showed that extrusion of dry-milled products reduced

fumonisin concentrations by 30–90% for mixing type extruders and by 20–50% for non-mixing extruders. Katta *et al.* (1998) examined the extrusion of corn grits on the stability of fumonisin B<sub>1</sub> using different temperatures and screw speeds. Both the barrel temperature and screw speed affected the loss of fumonisin in the extruded product with losses ranging from 34 to 95%. However, the loss ranged from 46–76% in those products with expansion and colour acceptable for commercial use.

Wolf-Hall *et al.* (1999) showed deoxynivalenol to be quite stable in heat-treated foods when the effects of high temperature and high pressure processing of foods spiked with deoxynivalenol were examined. However, Accerbi *et al.* (1999) showed that soaking wheat naturally contaminated with high concentrations of deoxynivalenol in sodium bisulphite solution reduced levels from 7.3 to 0.8 mg kg<sup>-1</sup> and then, after further extrusion, to 0.3 mg kg<sup>-1</sup>.

Conflicting results in the literature may result from a failure to control or report all the conditions used. For example, chemical breakdown in the extrusion process is related to time, so that the loss of mycotoxin will depend on the residence time of the material in the extruder. The presence of minor ingredients or additives may also have a significant effect. These details are often missing from reports.

The present work forms part of a much larger study aimed at examining the fate of fusarium mycotoxins during milling and selected food processing.

**Materials and methods**

*Wholemeal wheat containing mycotoxins*

A number of wheat samples harvested from experimental plots were analysed for mycotoxins and, on the basis of the results, were mixed to provide a batch of about 200 kg of wheat with DON content of approximately 1000 µg/kg. This wheat also contained ZON at about 300 µg/kg, nivalenol (NIV) at about 200 µg/kg and lower concentrations of other related trichothecenes. The whole wheat was milled at the Campden and Chorleywood Food Association (CCFRA) using a pilot-scale Buhler milling rig as used in the Chorleywood Bread Process, but collecting all the milled fractions so as to produce wholemeal wheat flour. The Chorleywood Process was developed in 1961 by cereal scientists (Chamberlain *et al.* 1962, Axford *et al.* 1963).

The flour was mixed and blended for two hours and placed in a series of polythene bags as described in a previous study on milling and baking (Scudamore et al. 2003). The moisture content of the wholemeal wheat flour produced was approximately 13%. The wheat in these bags was individually mixed again and samples of about 250 g were taken at random from each bag. The samples were then analysed separately for DON, ZON and NIV. The contents of each bag were finally recombined and re-mixed in two large batches of 65-70kg for convenience of handling during the study. After a final blending, samples from each batch were analysed again for mycotoxin content. The wholemeal wheat was calibrated for flow rate and moisture prior to setting up the processing conditions for extrusion cooking.

#### *Processing conditions*

An APV twin screw extruder model MPF 50D (supplied by APV Peterborough, UK) was set up with a screw comprised of APV type elements to form sections for conveying, mixing in water, reverse pumping in a shear zone and final high pressure pumping. The arrangement of the screw elements given in Table 1 shows a section of feed screws conveying the powder into a short mixing section of paddles and on via conveying section to the reversing paddles section. Resistance from the reversing paddles causes the section to fill and generate a pressure via the twin start screws. This in turn generates heat and melts the crystalline structures in the starch granule so that a melt fluid forms. In the final screw section the hot fluid is sealed under pressure and the water superheated to more than 140°C and as it returns to atmospheric pressure outside the barrel the water vaporises to expand the extrudate.

The die plate contained two circular dies of 3mm diameter and 4mm land length. At the start of the extrusion cooking run the barrel heaters were set at 0/50/70/120/140°C and adjusted to raise the barrel temperature to 160 and then 180°C by changing the last two sections. The powder and water feed rates were adjusted to the computed settings on the plan to run at 700g/min for 15, 18 or 21% moisture w/w. The screw speed was held at 300rpm for all runs.

#### *Experimental plan*

The experimental plan involved a sequential change in extruder conditions, Table 2, commencing at 140°C and finishing at 180°C. The extruder was run up to a steady

state at each change over and held for 2 minutes to stabilise the extrusion before a sample was collected for 2.5 minutes representing about 1.5 to 2 kg of extrudate. This was allowed to cool before packing. Logging data was taken to confirm the target settings.

*Analysis*

Deleted: ¶

The methods of analysis used have been described previously but are summarized here.

*Extraction, clean-up and determination of mycotoxins*

All reagents were purchased from commercial sources and were of analytical grade. Solvents were of HPLC grade. Mycotoxin standards of DON, NIV, and ZON were purchased from Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK. DON, NIV and ZON were quantified prior to use by UV spectrometry, prepared 3-monthly. Trichothecene charcoal/alumina clean-up columns (Tricho columns®) and zearalenone immunoaffinity clean-up columns (Zearalenone Easi-Extract®) were purchased from R-Biopharm Rhone Ltd., Glasgow.

The methods used for the trichothecenes and ZON are UKAS (ISO 17025) accredited and have performance characteristics that meet the requirements for analytical methods for mycotoxins stipulated by CEN (CEN 1999) and the EC.

Prior to analysis samples were ground to 1 mm particle size in a laboratory mill and mixed for 1 hour on either a Winkworth cone blender or Pascall laboratory scale powder mixer depending on sample size.

*DON and NIV*

Ground samples (20 g) were extracted with 100 ml acetonitrile/water (84:16) by shaking for 2 hours on a wrist action shaker. An aliquot of the filtered extract (5 ml) was transferred to a glass tube supplied with the charcoal/alumina clean-up column. The plunger of the column was inserted into the tube and the column was slowly pushed through the extract at the rate of about 1 ml per 30 seconds until approximately 3 ml of cleaned up extract was collected. An aliquot (2 ml) of cleaned up extract was transferred to a 4 ml vial and taken to dryness under nitrogen using a

heating block set at approximately 50°C. The residue was derivatised to form the trichothecene-trimethyl silyl (TMS) derivatives and determined by GC/MS operating in selected ion mode, using 4 ions for confirmation. Trichothecenes detected are DON, 3AcDON, 15AcDON, NIV, DAS, FUSX, T2, and HT2.

### ZON

The method used was that of MacDonald et al, (2005). Ground samples (25 g) were extracted with 100 ml of acetonitrile/water (75/25) by blending for 3-5 minutes and the extract was filtered. Twelve ml of the extract was diluted with 88 ml of Phosphate Buffered Saline (PBS) solution. An aliquot (50ml) of the diluted extract was passed through EASI-EXTRACT ZON immunoaffinity column (R-Biopharm Rhone) ) at a rate of no more than 3 ml/minute. The column was washed with 20 ml water and dried by passing 3 ml of air through the column. ZON was eluted at slow speed (1-2 drops per second) with 1.5 ml of acetonitrile, followed by 5 ml of air to ensure all eluate was collected. An aliquot (1 ml) of the eluate was diluted with water (2 ml) and the diluted extract injected onto HPLC. These operations were carried out automatically by a Gilson ASPEc system. ZON was detected by HPLC with fluorescence detection. Liquid chromatography was performed on either a Phenomenex ODS3-Prodgy 5 µm particle size 15 cm x 4.6 mm i.d., 250A pore size or a Spherisorb ODS2-Excel 5 µm particle size 25 cm x 4.6 mm i.d., 250A pore size operated at 1.0 ml min<sup>-1</sup> with water : acetonitrile (45:50) as mobile phase. The excitation wavelength of the fluorescence detector was set at 275 nm and the emission wavelength was 450 nm. Sample volumes of 400 µl were injected.

### *Method validation and quality control*

UKAS accreditation ensures that methods are valid for the tasks they are performing, but do not provide an absolute measure of accuracy. Under the Laboratory Quality System a protocol for internal and external proficiency test schemes participation is defined and the laboratory demonstrates on-going precision and accuracy. This includes analysis of in-house reference material and participation in sample testing schemes such as FAPAS ([www.fapas.co.uk/fapas.cfm](http://www.fapas.co.uk/fapas.cfm)) and collaborative projects. On-going control of the methods was monitored using in-house naturally contaminated

reference materials and spiked samples. Typical mean recoveries for each toxin ranged from 70 to 110 %.

*Recovery, Limit of Detection and Determination*

All analyses were conducted with a spiked sample, i.e. a known amount of toxin was added to a sample of ground wheat each day prior to extraction, clean-up and detection for each batch of samples. These samples are used to access recovery, and recoveries of 70-110% were considered acceptable. The spike level was 500 µg/kg for each trichothecene and 50 µg/kg for ZON.

The limit of detection and limit of determination was 20 µg/kg and 50 µg/kg respectively for each trichothecene and 2 and 5 µg/kg for ZON. The limit of detection is defined as 3 times the electronic baseline noise and the limit of determination as the lowest level at which validation has been performed. After analysis samples were retained and stored at -20°C.

*Measurement uncertainty*

The analytical results have a variability known as measurement uncertainty. For any analytical method each result is reported as the best estimate for the sample and it is always qualified by a measurement uncertainty, e.g. x microgram/kg ± y microgram/kg. For this survey the calculation of the measurement uncertainty was carried out using in-house data, performance in international collaborative trials and FAPAS, thus incorporating repeatability and reproducibility data. The expanded measurement of uncertainty was calculated using a standard coverage factor of 2, equivalent to a confidence of approximately 95% that the actual level of the mycotoxin being measured lies within the quoted range. The assessment of measurement uncertainty was targeted at samples containing mycotoxins at a range of levels. The expanded measurement of uncertainty for these analyses was assessed to be ±34% at a level of 226 µg/kg ZON in wheat and ± 49% at a level of 500 µg/kg for DON and ±42% at a level of 500µg/kg for NIV.

*Results from check trials*

Acceptable results have been achieved in Series 22 of the FAPAS Scheme for deoxynivalenol and ZON in cereals during the period of the study (rounds 23 to 33,

January 2006 – February 2007) as they were well inside the limits for Z scores of  $\pm 2$  considered acceptable.

#### *Moisture content determination*

The moisture contents of the extrudates were measured by taking a 5g-sample and heating in an oven at 105°C for 16 h.

## **Results and Discussion**

### *Homogeneity of the wholemeal wheat flour*

The method for testing the homogeneity of the mycotoxin concentrations in wholemeal flour was described in the method section. Table 3 gives the data showing the homogeneity of the mycotoxin distribution found in the flour in samples taken at random from the two batches of wheat flour used, after the final mixing. It presents the individual and mean values for DON, NIV and ZON and the percentage standard deviation values. These were considered satisfactory for the study to proceed especially as the variability of mycotoxin concentrations in the experimental samples is likely to be further minimised when the flour is mixed again in the supply hopper and during the extrusion cooking.

The mean value for DON in this consignment is thus close to 1000 µg/kg. This compares with a maximum level of 750 µg/kg for DON in flour and 500 µg/kg in finished consumer products set by the EC from 1 July 2006. Thus the flour examined here should represent closely what would occur in flour containing DON at that value. From the 1 July 2006 values for ZON of 75 µg/kg in wheat flour and 50 µg/kg in specified consumer products have been in place within the European Union. This compares with the value around 300 µg/kg in the current flour. There are no proposals for limits for NIV but in view of its toxicology any future legislation might be at or below the limits for DON.

### *Analysis*

The analytical methods for the analysis of DON, NIV and ZON have proven to be accurate and reliable in other studies and have been widely used for wheat and milled wheat products. In this study all recoveries fell within the accepted range of 70-110%.

Sample results were corrected for the values found in spiked samples included with each analytical batch. Performance data presented here met the criteria of recovery and variability considered acceptable (CEN 1999).

*Extrusion*

The moisture contents of the wheat flour were  $13.04 \pm 0.01$  and  $13.34 \pm 0.02$  % w/w for batches 1 and 2, respectively. It gave a linear flow relationship ( $R^2$ ) between the speed of K-tron feeder and the weight delivered into the barrel, mass of flour g/min =  $65.1x + 97.9$  where x is the speed of the K-tron.

The extrusion cooking process was run under controlled conditions for all the samples in the order shown in Table 2. Wholemeal wheat from batch 1 was used to examine the effects of experimental conditions 1-12 and from batch 2 for conditions 13-18. The running conditions were matched in all the samples for feedrate and moisture and the temperatures were set as the barrel temperatures, 140, 160 and 180°C. The values for the feed rate during the study are also given in Table 2 and indicate that this remained quite constant throughout. This is important because if any loss of mycotoxins occurs because of chemical breakdown the time spent in the extruder becomes crucial and would introduce yet another factor if not controlled. The extruder pressure at the die depends on the conditions but increases with temperature. The SME is a measure of the energy input to the wheat extrudate and values for specific volume indicates the degree of expansion resulting from each different condition. The largest expansion occurred at low moisture content and high temperature.

Tables 4-6 give the mean change in concentration as the % remaining for DON, NIV and ZON respectively during extrusion of wholemeal wheat flour (positive values indicate an increase in mycotoxin concentration). All results are adjusted for the moisture content of the input wheat and that of the final extrudate. Two samples were taken at each experimental set of conditions. The standard deviation between replicate pairs of extrudates is small and suggests that significant loss of DON (Table 4) occurred only in the wheat at 15% moisture content although increasing temperature did not increase loss. If DON is being lost through chemical reaction this should increase with increasing temperature. The reason for this result is unclear but could

1  
2 indicate that the loss is due either to binding to the wheat constituents or to poor  
3 extractability from the extruded products. At each temperature setting the loss was  
4 least in the samples extruded at a moisture content of 21%. The variability between  
5 pairs of samples was very small as shown by the low values for % SD so that these  
6 differences are real. The levels of DON were just above the maximum permissible  
7 legal limit set within the EC.  
8  
9

10  
11 NIV is a compound structurally closely related to DON but is present here at  
12 lower concentrations which may account for rather more variation between sample  
13 pairs. At each temperature the lowest values were obtained with the lowest moisture,  
14 Table 5. However, under nearly every condition tested the concentration of nivalenol  
15 after extrusion had apparently increased. The reason for this is unclear but could be  
16 that the extrusion process is releasing 'bound' nivalenol. With 4 free hydroxyl groups  
17 NIV is more polar than DON and this might be a significant factor.  
18  
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23 The results for ZON, Table 6 differ from those for DON and NIV in that the lowest  
24 results were in the driest samples although virtually all the ZON was recovered or  
25 slightly increased in value. ZON is much less polar than NIV or DON and this may be  
26 a factor. It is difficult to explain some of the results for these fusarium mycotoxins.  
27 However, they are all quite resistant to loss during extrusion. The apparent increase  
28 under some conditions needs further studies to confirm this. The trend for residual  
29 ZON values to tend to increase going from 140 to 180 degrees is contrary to what  
30 would be expected from chemical degradation. This is also seen for NIV but not to  
31 any extent for DON. The answer to these changes may lie with the efficiency of the  
32 extraction solvent used for removing mycotoxins in the extruded materials or that  
33 bound mycotoxins are being released by the extrusion conditions - or a combination  
34 of both.  
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#### 43 *Assessment of variability*

44 Variability within the results can arise due to several factors. The homogeneity of the  
45 mycotoxin distribution in the wholemeal flour used, the analytical method, changes in  
46 moisture during the extrusion process, the residence time in the extruder and the  
47 efficiency of the solvent in extracting the mycotoxins from the range of extruded  
48 products formed. Data are presented for the homogeneity of the flour and the  
49 performance of the analytical method. The residence time within the extruder is  
50  
51  
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standardised and results are corrected for moisture changes. Each extruded flour sample is then compared directly with the sample of flour taken immediately before extrusion.

One area of uncertainty remains regarding the effectiveness of extraction as all results for recovery are based on spiking. No certified reference materials exist for these mycotoxins in extruded products so this must be recognised in assessing the findings from this study. The conditions within the extruder produce a range of materials with very different physical characteristics to those of wheat flour. The products include light, highly expanded materials and hard, dense products, each physically different in structure to the wheat flour. This raises the possibility that the efficiency of solvent extraction and recovery could vary from product to product.

**Conclusions**

Temperature does not appear to significantly degrade any of the three mycotoxins under the extrusion conditions studied. Moisture content appears to be more important than temperature as low moisture tends to result in lower values for DON (and possibly for NIV) at all temperatures. Because loss does not appear to be affected by temperature it is probably due to binding to the wheat and/or poor extractability of the analytical solvent. Current EC legislation relies on processing to reduce DON concentrations from 750 µg/kg to 500 µg/kg and a reduction for ZON from 75 µg/kg to 50 µg/kg. This reduction appears unlikely to be achieved for some consumer products such as snacks made by a simple extrusion of wheat flour (and probably also maize flour).

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extrusion was carried out at the Campden and Chorleywood Food Research Association, Chipping Campden, UK.

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Table 1. Technical detail of the arrangement of screw elements

Screw	Length, mm
Feed screw 1D	304.8
Seven forwarding paddles at 45°	88.9
Single lead screw	152.4
Two paddles at 90°	25.4
Twin start lead screw	50.8
Two paddles 90°	25.4
Four reversing paddles at 45°	50.8
Single lead screw	50.8
Total length	749.3

Table 2. Sequence of extruder conditions, samples taken, and related experimental parameters at a screw rate of 300rpm and nominal feedrate of 700g/min.

Sample order	Temperature,	Feedrate,	SME*,	Pressure,	Moisture, %		Specific volume
	°C	g/min	kJ/kg	MPa	Wheat flour	Extrudate	L/kg
1	140	704	490	1.8	21	9.29	5.81
2	140	703	466	1.7	21	9.48	5.73
3	140	709	574	2.2	17	7.38	7.37
4	140	698	571	2.0	17	7.51	7.30
5	140	703	615	3.7	15	4.22	7.67
6	140	682	583	3.6	15	4.61	7.52
7	160	716	539	3.5	15	4.38	7.72
8	160	704	537	3.5	15	4.17	7.52
9	160	692	526	1.8	17	7.51	6.55
10	160	675	460	1.8	17	7.74	6.61
11	160	705	442	1.8	21	8.61	5.61
12	160	705	441	1.6	21	8.81	5.73
13	180	705	374	1.6	21	8.81	7.42
14	180	705	374	1.6	21	8.95	7.25
15	180	702	456	2.1	17	7.54	5.80
16	180	698	466	2.1	17	7.37	5.77
17	180	706	534	2.8	15	4.30	8.31
18	180	706	488	2.7	15	4.05	8.38

\* SME= specific mechanical energy

Table 3. Mycotoxin concentrations and homogeneity in wholemeal wheat flour used for extrusion study

Sample number	Mycotoxin, $\mu\text{g/kg}$		
	DON	NIV	ZON
<b>BATCH 1</b>			
1a	1144	223	267
1b	1289	179	266
1c	1280	185	273
1d	1368	232	280
1e	1237	274	277
Mean	1264	218	273
SD	81.9	38.3	6.1
% SD	6.5	17.5	2.2
<b>BATCH 2</b>			
2a	856	198	309
2b	1124	213	314
2c	974	202	349
2d	1042	198	354
2e	848	173	360
Mean	969	208	337
SD	119.2	14.6	23.8
% SD	12.3	7.4	7.1

Table 4. Effect of extrusion on concentrations of deoxynivalenol (1264 or 969 µg/kg) corrected for moisture content changes.

Extrusion Temperature, °C	Wheat Moisture, %	Sample	DON, Surviving, %	% loss	Sample pairs, % SD	
140	21	a	96.3	3.2	0.73	
		b	97.3			
	17	a	90.7	6.0	4.96	
		b	97.3			
	15	a	79.5	18.9	2.88	
		b	82.8			
	21	a	102.2	+0.2	2.82	
		b	98.2			
160	17	a	96.2	+0.3	5.78	
		b	104.4			
	15	a	75.8	23.4	1.48	
		b	77.4			
	21	a	101.5	0.4	1.34	
		b	99.6			
	180	17	a	98.3	+1.0	3.85
			b	103.8		
15		a	81.9	20.5	4.18	
		b	77.2			
Mean, % SD					3.11	

Table 5. Effect of extrusion on concentrations of nivalenol (197 µg/kg), results corrected for moisture content changes.

Extrusion Temperature, °C	Wheat Moisture, %	Sample	NIV, Surviving, %	% change	Sample pairs, % SD	
140	21	a	105.1	+6.95	3.73	
		b	110.8			
	17	a	114.6	+8.85	7.47	
		b	103.1			
	15	a	96.5	-3.55	0.07	
		b	96.4			
	160	21	a	128	+24.15	4.39
			b	120.3		
17		a	110.5	+12.9	1.77	
		b	113.3			
15		a	106.0	-1.9	11.39	
		b	90.2			
180		21	a	147.9	+39.9	8.09
			b	131.9		
	17	a	142.9	+44.9	1.95	
		b	146.9			
	15	a	132.3	+33.1	0.32	
		b	132.9			
	Mean % SD					4.35

Table 6. Effect of extrusion on concentrations of zearalenol (305 µg/kg), results corrected for moisture content changes.

Extrusion Temperature, °C	Wheat Moisture, %	Sample	ZON, Surviving, %	% change	Sample pairs, % SD	
140	21	a	86.3	-17.4	6.25	
		b	79.0			
	17	a	106.2	-2.9	13.17	
		b	88.1			
	15	a	113.9	+15.35	1.78	
		b	116.8			
	160	21	a	95.8	+2.45	9.18
			b	109.1		
17		a	110.7	+6.15	6.06	
		b	101.6			
15		a	124.3	+4.8	26.31	
		b	85.3			
180		21	a	95.7	+2.45	9.32
			b	109.2		
	17	a	105.5	+10.2	6.70	
		b	116.0			
	15	a	104.7	+7.75	4.00	
		b	110.8			
	Mean % SD					9.20