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Effects of deoxynivalenol and deepoxy-deoxynivalenol on bovine ovarian theca cell function

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Résumé

La mycotoxine déoxynivalénol (DON) et son métabolite déépoxy-déoxynivalenol (DOM-1) ont des effets significatifs sur la modification de la fonction des cellules thècales de l'ovaire bovin. L'objectif de cette étude était d'identifier les différentes voies de signalisation impliquées dans le mécanisme d'action de DON et DOM-1 par la spectrométrie de masse. Méthodes: Les cellules thécales de l'ovaire bovin ont été récoltées à partir des vaches adultes, indépendamment du stade du cycle œstral, et ont été cultivées à une densité de 500000 cellules viables dans 1 ml de milieu de McCoy pendant 5 jours. Les cellules ont ensuite été traitées au jour 5 de la culture avec 1 ng/ml de DON ou DOM-1 pendant 30 minutes et des échantillons cellulaires de protéins totales ont été préparés pour spectrométrie de masse. Résultats: la spectrométrie de masse a montré que DON et DOM-1 induisent une surexpression simultanée de ERK1/2, MAPK14 (p38alpha) et MAPK13 (p38delta). La spectrométrie de masse a également indiqué que 94 peptides ont été surexprimés tels que GNGT1, EDN1 et YWHAB. Ils régulent la plupart des voies de prolifération des cellules et sont impliqués dans la biosynthèse des lipides et des glucides. Néanmoins, 255 peptides ont été régulés à la baisse, tels que CALR3, PTGES3, RAD21, ACVR2B et TGFBR1 dont leurs activités sont principalement l'activation ou la désactivation des processus apoptotiques, et le métabolisme du glucose et de la choline. Nos résultats montrent que DON et DOM-1, à une dose de 1 ng/ml, ont le potentiel de stimuler la surexpression de MAPK distinctes et réguler négativement les voies de signalisation spécifiques qui stimulent la prolifération les cellules de la thèque de l'ovaire de bovin.

Mots-clés : phosphoprotéome, les cellules de la thèque, déoxynivalénol, déépoxy-déoxynivalenol, protéines kinases activées par des mitogènes, la prolifération

Abstract

The mycotoxin deoxynivalenol (DON) and its metabolite deepoxy-DOM-1 have significant effects on bovine ovarian theca cell function. The objective of this study was to identify different signaling pathways involved in the mechanism of action of DON and DOM-1 by mass spectrometry. Methods: bovine ovarian theca cells were harvested from adult cows independently of the stage of the estrous cycle, and were cultured at a density of 500000 viable cells in 1 ml McCoy's medium for 5 days. The cells were then treated on day 5 of culture with 1 ng/mL DON or DOM-1 for 30 minutes and total cell protein was collected for mass spectrometry. Results from mass spectrometry showed that both DON and DOM-1 induce simultaneous upregulation of ERK1/2 , MAPK14 (p38alpha) and MAPK13 (p38delta). Mass spectrometry also indicated that 94 peptides such as GNGT1, EDN1 and YWHAB were upregulated. They mostly regulate cell proliferation pathways and are involved in biosynthesis of lipid and carbohydrates. Nevertheless, 255 peptides such as CALR3, PTGES3, RAD21, ACVR2B and TGFBR1 were downregulated whose activities are mainly activation or deactivation of apoptotic processes, and glucose and choline metabolism. Our findings show that both DON and DOM-1 at least at a low dose (1 ng/ml) have the potential to stimulate upregulation of distinct MAPKs and downregulate specific signaling pathways that stimulate bovine ovarian theca cell proliferation.

Keywords : phosphoproteome, theca cells, deoxynivalenol, deepoxy-deoxynivalenol, mitogen-activated protein kinases, proliferation

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List of abbreviation

ACLY: ATP citrate synthase

ACVRB: activin receptor type-2 B

AFB1: aflatoxin B1

CALM4: calmodulin

CHORDC1: cystein and histidine-rich domain-containing protein 1

CGCs: cumulus granulosa cells

COCs: cumulus oocyte complexes

CYP11A1: cytochrome p450 cholestrol side-chain cleavage

CYP17A1: cytochrome P450 17α-hydroxylase

DIA: data independent analysis

DOM-1: deepoxy-DON

DON: deoxynivalenol

E2: estradiol

EDN1: endothelin 1

EIF5A: eukaryotic translation initiation factor 5A-1

ENPP6: ectonucleotide pyrophosphatase

ERK1/2: extracellular regulating kinase 1/2

FB1: fumonisin B1

FDA: food and drug administration

FSH: follicle stimulating hormone

GIT: gastro-intestinal tract

GNGT1: guanine nucleotide-binding protein G subunit gamma-T1

GnRH: gonadotropin releasing hormone

HRAM: high-resolution, and accurate-mass

HSD3B: 3β-hydroxysteroid dehydrogenase

HSD17B: 17β-hydroxysteroiddehydrogenase

HSP90: heat shock protein 90

IOI: interovulatory interval

JNKs: cJun NH_2-terminal kinases

LH: luteinizing hormone

MAPKKs: mitogen-activated protein kinase kinases

MAPKs: mitogen-activated protein kinases

MDH1B: malate dehydrogenase 1β

NDUFB3: NADH dehydrogenase [ubiquinone]1-β-subcomplex subunit 3

OMM: outer mitochondrial membrane

P4: progesterone

p38: protein kinase p38

PBS: phosphate-buffered saline

PDIA3: protein disulfide-isomerase A3

PTGES3: prostaglandin E synthase 3

RAD21: double-strand-break repair protein rad 21 homolog

RANBP2: E3 SUMO-protein ligase RanBP2

RSR: ribotoxic stress response

StAR: steroidogenic acute regulatory protein

START: StAR-related lipid transfer

STAT: signal transducers and activators of transcription 3

TCs: theca cells

TCA: tricarboxylic acid

TGFα: tumor growth factor α

TGFBR1: transforming growth factor-β receptor type 1

Th2: T-helper

TOMM5: mitochondrial import receptor subunit TOM5

YWHAB: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein or 14-3-3 protein β/α

ZEN: zearalenone

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Introduction

Fertility in dairy cows has decreased worldwide over the last several decades. This decline results from genetic selection for milk yield, and is compounded by a multitude of health, physiological, and management problems. To improve reproductive function while maintaining high milk yield, nutritional strategies are applied that provide signals to stimulate reproductive processes without decreasing milk production (Chagas et al., 2007).

The most common mechanisms that affect fertility in current dairy cows are anovulation, anestrus (due to failure to display estrus), abnormal and irregular estrous cycles, decline in embryo implantation and development and uterine/placental abnormalities. To improve fertility, strategies such as controlled breeding programs, using high fertility sires and uterine health surveillance are applied. However, this decline in fertility may be permanently solved with a thorough health-check system with special attention being paid to the function of the ovarian-uterus-hypophysis axis. The ovary plays a key role in the development of the female germ cell, the oocyte. The oocyte is developed and matured within special structures in the ovary called follicles and any disruption in normal follicle function will result in significant fertility issues (Lucy, 2006).

Ovarian follicle growth is regulated by precise interactions between three cell types within the follicle: theca cells, granulosa cells and the oocyte. Theca cells provide structural support for the follicle, and provide nutritional, paracrine and endocrine support for granulosa cells, including the secretion of androgens. Granulosa cells reside in an avascular compartment, and are essential for follicle growth and oocyte development. Follicle growth is dependent on estradiol secretion by granulosa cells, and only healthy granulosa cells convert thecal androgen into estradiol. Therefore the health of both granulosa and theca cell layers is crucial for fertility (R. Scaramuzzi et al., 2011).

Follicle development can be affected by numerous environmental factors, including toxins of plant or man-made origin. One major source of toxins is fungi associated with animal feed. Mycotoxins have been reported to reduce fertility in pigs and poultry, and to lead to major economic losses. The effects of zearalenone on pig production has been widely reported

and the mechanisms of action have been elucidated. Another major mycotoxin is deoxynivalenol (DON), which also affects pigs and poultry, and acts by inhibiting protein translation. In ruminants, DON is metabolized in the rumen to deepoxy-DON (DOM-1), which is substantially less toxic than DON. However, the impact of DON and of DOM-1 on ovarian function in ruminants has not been well explored, and there are no reports on the effects of any mycotoxin on theca cell function.

The objective of the present work was to shed light on the mechanism of action of DON and DOM-1 in bovine theca cells. To do so, we used a proteomic approach to determine which intracellular pathways are activated or deactivated by these two mycotoxins. The following section describes the physiology of ovarian function and the biochemistry and biopathology of major mycotoxins occurring in North America.

Chapter 1 : Literature review

1. The ovaries

The ovary is the main female reproductive organ and has two important functions: producing the female germ cell (the oocyte) and producing the hormones estrogen and progesterone. Each cow has two ovaries that are oval or bean-shaped. They are 1-1.5 inches long and are located in the abdominal cavity. The main function of the ovaries, the maturation and release of oocytes, is achieved via the production of several steroidal and nonsteroidal hormones. These hormones act on various target organs, including the uterus, vagina, fallopian tubes and mammary glands, leading to the female phenotype. The secretion of ovarian hormones is precisely regulated by the hypothalamic-pituitary axis. The complex interactions between hypothalamic, pituitary and ovarian hormones are responsible for the regular and predictable ovulatory cycle and fertility in females (Gupta & Chia, 2013).

The ovary contains several thousands of small structures called primordial follicles. Each follicle has a germ cell surrounded by a single layer of squamous cells. This germ cell has the potential to mature into a fertilizable oocyte if the follicle activates and starts to grow, although most of the primordial follicles never develop. When a primordial follicle activates, the squamous cells become cubic and the follicle is then termed primary. These cells divide to form multiple layers of cells, which are called granulosa cells, and the follicle is named a secondary follicle. As the follicle enlarges, a cavity forms within the granlosa cell layer, and the follicle becomes an antral follicle (Shah & Nagarajan, 2013).

As the follicle and cavity grow, the oocyte becomes attached to the back of the follicle (opposite the ovulation site) by a mass of cells termed the cumulus oophorus. As the follicle reaches maximal size, the side opposite the oocyte becomes very thin. Once the follicle reaches this state it is called a Graafian follicle. At ovulation, this surface ruptures to release the oocyte (Whittier, 1993).

1.1. Structure

Each follicle consists of an oocyte surrounded by a glycoprotein layer, the zona pellucida, and some layers of somatic granulosa cells (GCs). The granulosa cell layer surrounding the oocyte forms the cumulus oophorus. The theca layer surrounds the granulosa cells, and possesses two sublayers: the theca interna and theca externa. Figure 1 illustrates the structure of a mature pre-ovulatory follicle in which the oocyte is surrounded by different cellular layers.

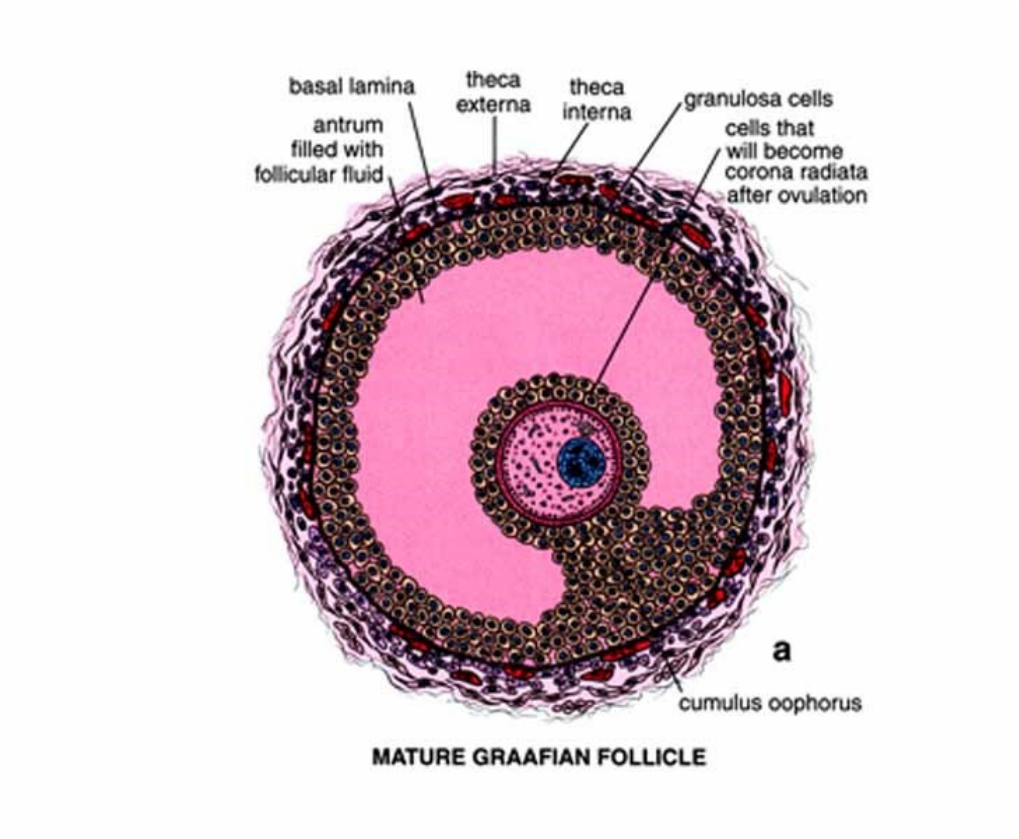

Figure 1. Structure of a mature graafian follicle of mammals. This follicle diagram identifies the location of the oocyte, cumulus oophorus, antrum, corona radiata, granulosa cells and the theca cells. This structure represents the pre-ovulatory status of a mature follicle that eventually ruptures to release oocyte (Baerwald, Adams, & Pierson, 2003).

1.1.1. Oocyte

A mature, good quality oocyte is identifiable by a perfectly spherical shape, a regular zona, and a translucent, homogeneously coloured cytoplasm without inclusions. The oocyte is embedded in a well-developed cumulus mass and surrounded by a layer of cumulus cells called the corona radiata. The health of the oocyte is critical for the development of healthy embryos. The developmental competence of the oocyte is progressively acquired during oogenesis through a range of cellular and molecular events that provide the oocyte with the ability to complete meiosis, become fertilized, undergo preimplantation development, and accomplish specific postfertilization stages as a consequence of the action of stored maternal genes and proteins (Coticchio et al., 2004).

1.1.2. Granulosa cells

GCs are closely associated with the developing oocyte in the ovary of mammals. The major functions of granulosa cells include the production of progesterone and estradiol, as well as growth factors thought to interact with the oocyte during its development. The sex steroid production is achieved by the action of follicle–stimulating hormone (FSH) stimulating granulosa cells to convert androgens (coming from the theca cells) to estradiol (E2) by the enzyme cytochrome P450 aromatase, particularly during the follicular phase of the estrous cycle. However, after ovulation the granulosa cells turn into lutein cells that produce progesterone (P4). The main role of progesterone is to maintain pregnancy and it causes the production of a thick cervical mucus that inhibits entry of sperm into the uterus. It has been found that coupling between GC is mediated specifically by connexin 43 and is essential for continued follicular growth, expansion of the GC population during early stages of follicular development, and that mutations in this gap junction lead to retarded oocyte growth and poor development of the zona pellucida (Garzo & Dorrington, 1984) .

1.1.3. Theca cells

Theca cells (TCs) are endocrine cells that play pivotal roles within the ovary by producing androgen substrates under luteinizing hormone (LH) control. They provide structural support of the growing follicle as it progresses through various developmental stages (Figure 2). They are highly vascularized and through this vascularization they provide the rest

of the follicle with essential nutrients and endocrine hormones from the pituitary gland. Theca cells are first observed once a follicle has two or more layers of granulosa cells, which is around the time when theca cells become LH responsive and steroidogenic enzymes are activated (Magoffin & Weitsman, 1994; Young & McNeilly, 2010).

Theca cells are highly differentiated with structural features characteristic of steroid-secreting cells including abundant mitochondria with vesicular cristae, agranular endoplasmic reticulum, and lipid vesicles. The mitochondria contains the first enzyme in the steroidogenic pathway, cytochrome P450 cholesterol side-chain cleavage (gene symbol, CYP11A), and the endoplasmic reticulum contains the remaining enzymes necessary to produce androgens. The lipid vesicles store the precursors for steroid hormone synthesis as cholesterol esters which are transported into the mitochondria by steroidogenic acute regulatory protein (Manna, Dyson, & Stocco, 2009).

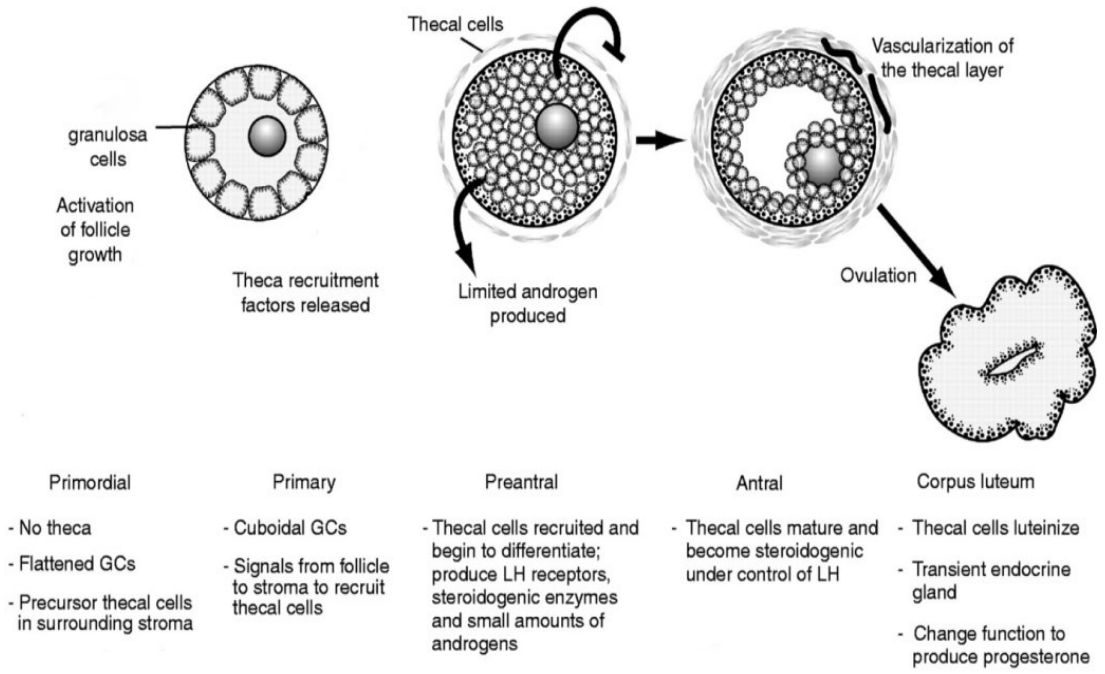

Figure 2. Illustration of theca cell development and function during folliculogenesis. Theca cells are absent from primordial follicles, and are recruited to growing preantral follicles. In antral follicles, the theca layer develops and becomes more steroidogenically active. After ovulation, the theca cells become part of the corpus luteum and secrete progesterone (Young & McNeilly, 2010).

1.2. Follicular growth and development (folliculogenesis)

The classification system for folliculogenesis has been well defined (see Figure 3). In brief, primordial follicles (type 1) are in the resting stage before being activated to start development and the oocyte is surrounded by one layer of flattened granulosa cells; type 1a are the follicles transitioning through to the primary (type 2) stage when the granulosa cells become cuboidal. Primary follicles have one layer of cuboidal granulosa cells, secondary follicles (type 3) have two to four layers of granulosa cells, large preantral (type 4) follicles

have four to six layers of granulosa cells, and antral follicles (type 5) have more than five layers of granulosa cells. It is after secondary follicle formation that the theca cells begin to emerge and form a layer around the granulosa–oocyte structure. Throughout folliculogenesis, the rates of atresia increase, and the early stages of folliculogenesis proceed very slowly therefore, most follicles are observed at early stages of development. At the antral stage, follicles become gonadotropin dependent and form large antral follicles (type 5+), most of which undergo atresia, and few are selected for ovulation (Edson et al. 2009).

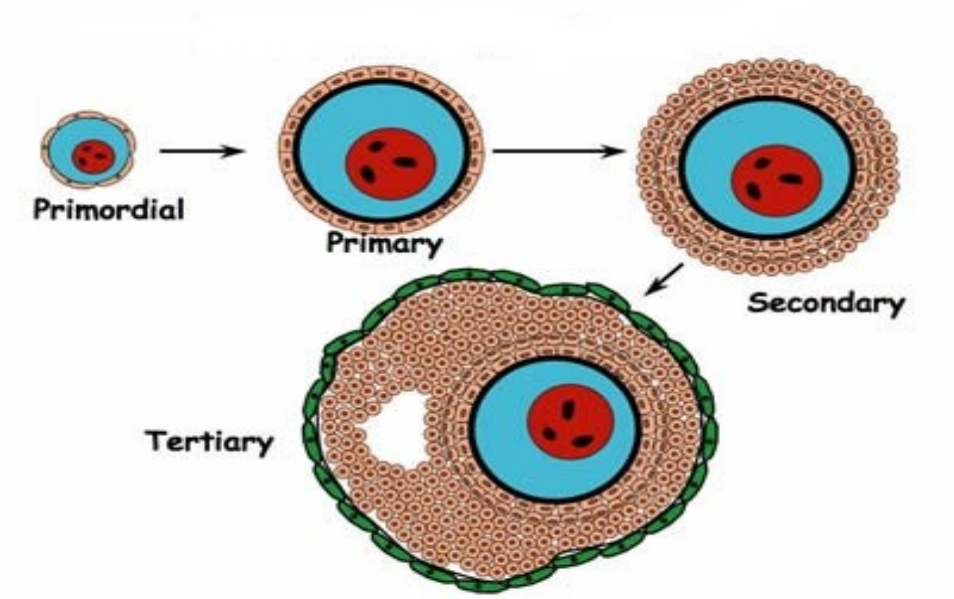

Figure 3. Folliculogenesis. Most follicles are in a resting state, and comprise the oocyte surrounded by a single layer of squamous pre-granulosa cells (primordial stage). When the follicle starts to grow, the granulosa cells become cubic, and the follicle is referred to as a primary follicle. The granulosa layer becomes multilayered as the secondary follicle forms. A tertiary follicle is one that has a thick granulosa layer and an antral cavity (Uda et al., 2004).

FSH plays a role in development of larger follicles beyond the antral stage. Though follicles beyond the antral stage are clearly gonadotropin dependent, the smaller preantral

follicles are gonadotropin responsive. FSH is not a survival factor for preantral follicles as it is for preovulatory and antral follicles, but it promotes follicle growth, granulosa cell division, and differentiation in follicles from the primary stage onward. The role of LH in folliculogenesis is more complex and somewhat contrasting. Theca cells constitutionally contain LH receptors; LH is capable of stimulating androgen production from TCs (to be transformed into estrogen by FSH-stimulated GCs) since fetal life, as shown by the elevated estrogen levels found in female neonates. Thereafter, because of prepubertal gonadotropin secretion quiescence, TCs remain unstimulated until the onset of puberty, when TC androgen production resumes (Filicori, 1999; McGee & Raj, 2015).

Follicular development occurs in a wave-like pattern in most domesticated animal species. Most studies suggest that 2 or 3 follicle waves occur during the oestrus cycle (Figure 4). This wave pattern is related to the length of interovulatory interval (IOI). An anovulatory wave emerges at the time of ovulation (day 0) followed by emergence of the ovulatory wave during the early-follicular phase with two follicular waves. In individuals with three waves, the first anovulatory waves emerges at day 0 and the second anovulatory wave emerges during the mid-to late-luteal phase, followed by the third and ovulatory wave emerging in the early-to mid-follicular phase (Yang, Yang, Li, & He, 2013).

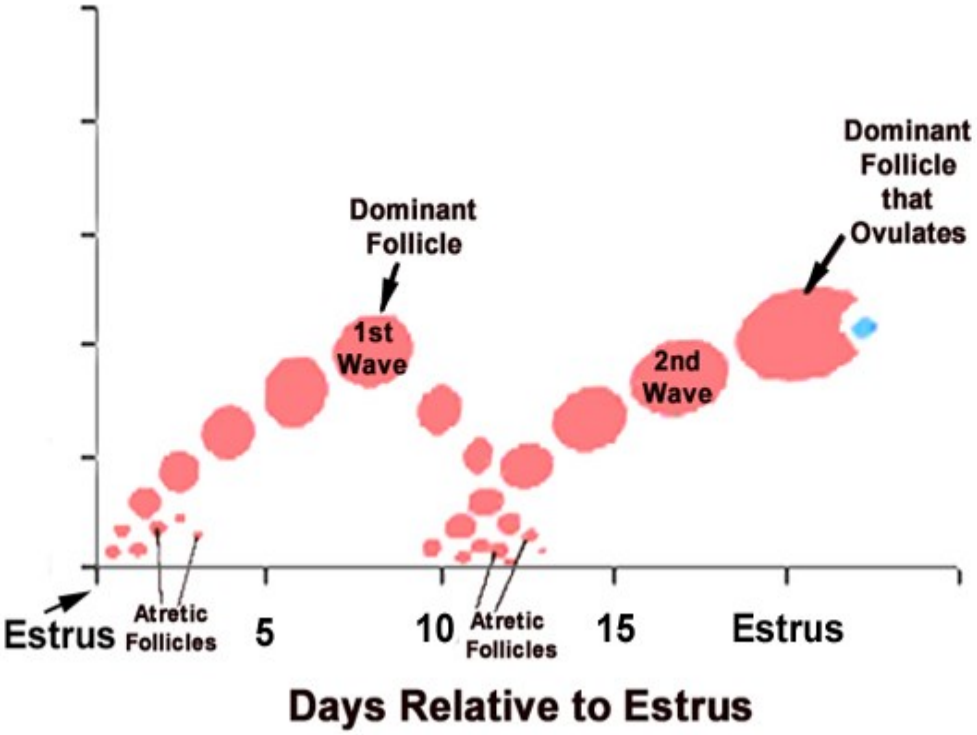

Figure 4. Follicular waves in cattle. After estrus, a cohort of follicles starts to grow, of which 1 becomes the dominant follicle and the remainder become atretic; this cohort forms the first follicle 'wave'. The first-wave dominant follicle regresses around day 8 of the cycle, and a new (2nd) wave emerges, the dominant follicle of which may become the ovulatory follicle at next estrus (R. J. Scaramuzzi et al., 2011).

During folliculogenesis, three major events take place: recruitment, selection and dominance. The recruitment begins with the growth of 8-41 small follicles between 3-4 mm, which continue to grow at a similar rate for several days. After this period, one of the follicles is selected and continues to grow until it becomes dominant, while the rest of the follicles become atretic and regress (Adams, Jaiswal, Singh, & Malhi, 2008).

1.2.1. Recruitment

The term recruitment has been given to the growth of follicles beyond the stage at which most follicles undergo atresia. During recruitment, follicles try to scale the "brick wall" that stands between them and ovulation. Recruitment is not a random or isolated phenomenon;

on the contrary, follicles seem to be recruited as groups or cohorts, suggesting that they have received a signal that allows them to continue growth and development rather than regress. The signal that stimulates recruitment appears to be a slight elevation in plasma FSH. In cattle, not only does a secondary surge of FSH on the day of ovulation precede the first follicular wave of the cycle, but also slight elevations in FSH have been shown to precede the second and third follicular waves of the cycle and the waves that occur in prepubertal animal (Webb et al., 2002).

1.2.2. Selection and dominance

The number of follicles recruited is usually greater than the typical number of ovulatory follicles for a given species. However, only a species-specific number of ovulatory follicles continues to grow for more than a few days and reaches ovulatory size. These follicles are called "dominant" follicles because it is believed that once they are selected, they in some way prevent further growth and differentiation of their sister, subordinate follicles and prevent further follicular recruitment. The secretion of feedback regulators, such as estradiol and inhibin, by the dominant follicle (or perhaps by the whole cohort of follicles during the first few days after recruitment) would cause a decrease in FSH to levels that would not support the further growth of subordinate follicles (Turzillo & Fortune, 1992).

In cattle, which develop dominant nonovulatory follicles as well as dominant ovulatory follicles, the differentiation of ovulatory follicles seems to occur in two stages. Dominant, nonovulatory follicles obtained during the course of the first wave of follicular development in cattle exhibit increased estradiol and decreased androgen in follicular fluid compared to subordinate follicles obtained from the same ovaries. Theca cells from dominant follicles secrete significantly more androgen and granulosa cells have a greater capacity to convert androgens to estradiol than follicular cells from subordinate follicles. Therefore, even dominant follicles that are selected during the luteal phase develop an enhanced capacity for thecal androgen and granulosa estradiol production. Presumably androgen is low in the follicular fluid of healthy dominant follicles because androgen secreted by the theca is used as a precursor for estradiol synthesis by granulosa cells. If progesterone secretion remains at

luteal levels, these dominant follicles do not continue to increase estradiol secretion (Adams, 1998).

Estradiol secretion decreases as follicle growth slows after ovulation due to decreasing aromatase activity in granulosa cells. This decrease in negative feedback from the ovary, as a dominant, nonovulatory follicle begins to regress, presumably allows the next small increase in basal FSH, which occurs too late to rescue the faltering dominant follicle, but induces the next round of recruitment (Dunlop & Anderson, 2014; Mesen & Young, 2015).

1.2.3. Atresia

Ovarian follicle development is tightly regulated by crosstalk between cell death and survival signals. Depletion of the ovarian follicular reserve starts during fetal life and continues throughout life. Only a small proportion of the primordial follicles will reach the ovulatory stage, while the rest of the follicles will undergo the degenerative process called atresia. In a natural estrous cycle in cattle, there is only one follicle that will be chosen to ovulate while the others will undergo atresia under the regulation of the hypothalamus-pituitary-ovary axis, intraovarian cytokines and gonadal steroids. Atresia is likely initiated by either loss of growth factor support, such as tumor growth factor α (TGFα) or FSH, or increased expression of 'death' factors such as Fas or Fas ligand (Dharma, Kelkar, & Nandedkar, 2003; Ulug et al., 2003).

2. Steroidogenesis

Steroidogenesis is the process by which cholesterol is converted to steroid hormones. Steroid hormones can be classified into five categories: glucocorticoids (cortisol), mineralocorticoids (aldosterone), androgens (testosterone), estrogens (estradiol and estrone) and progestins (progesterone). The first step in steroidogenesis takes place within mitochondria in which cholesterol is transported to the outer mitochondrial membrane. Steroidogenic acute regulatory protein (StAR), which is a member of a family of proteins that contain so-called “START” (StAR-related lipid transfer) domains, facilitates movement of cholesterol from the outer to the inner mitochondrial membrane. Steroidogenesis is initiated by mitochondrial cytochrome P450 cholesterol side-chain cleavage (CYP11A1) that cleaves the 20,22 bond of cholesterol to produce pregnenolone, and is the hormonally regulated, rate-

limiting step in steroidogenesis. Pregnenolone may then be converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B), found both in the mitochondria and in the endoplasmic reticulum (ER). Alternatively, pregnenolone is converted to 17α-hydroxypregnenolone by the enzyme cytochrome P450 17α-hydroxylase (CYP17A1), which is then converted to androstenedione by CYP17A1 and HSD3B. Androstenedione is then converted to testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B). Theca cells secrete androstenedione and testosterone, and GCs use CYP19A1 to convert androstenedione to estrone and testosterone to estradiol (Baker, Yaworsky, & Miller, 2005; Miller & Auchus, 2011). Figure 5 illustrates the process of steroidogenesis and the interaction between the theca and granulosa cells to produce steroids.

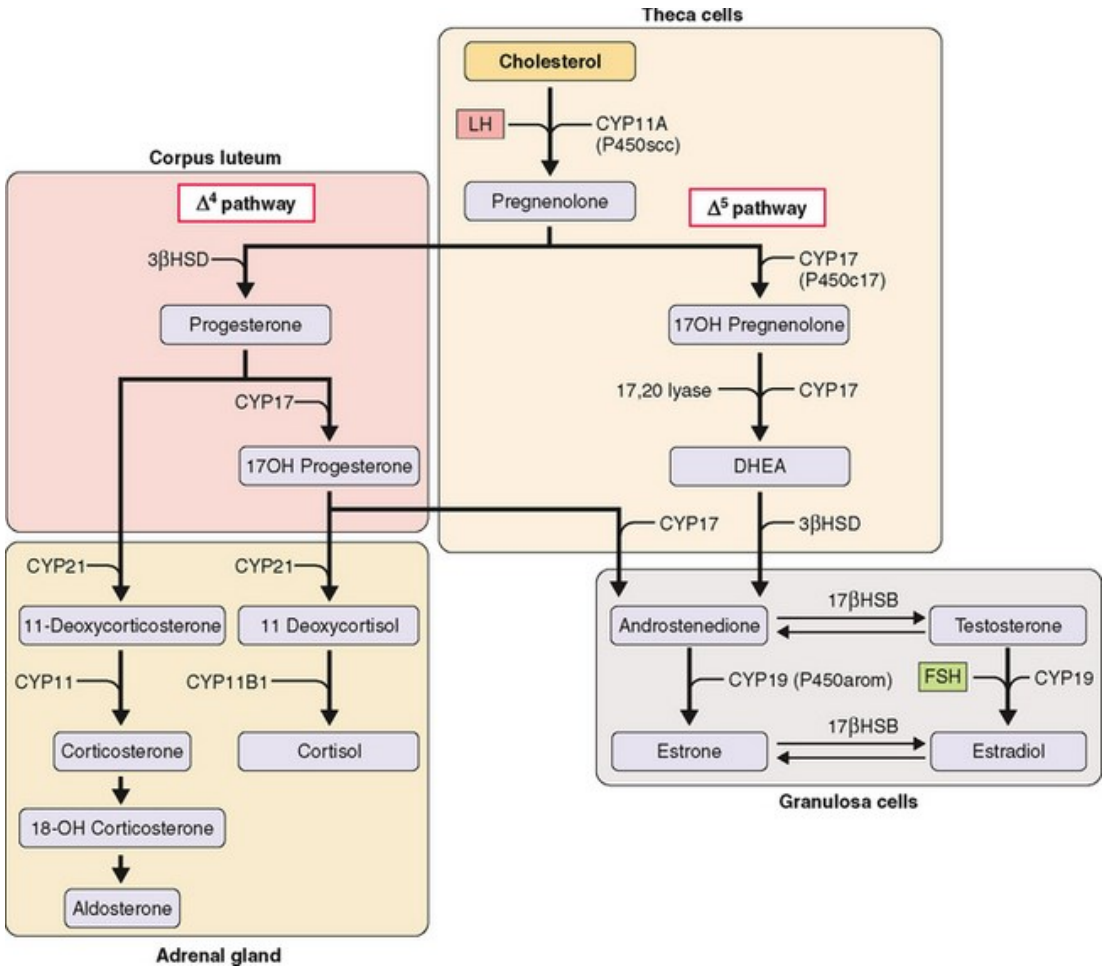

Figure 5. Steroid hormone biosynthesis pathways in the ovary. The Δ4 pathway converts pregnenolone to progesterone and is the main pathway in the corpus luteum. The Δ5 pathway converts pregnenolone to androgens and is the preferred pathway in the thecal cells. Granulosa cells are not able to synthesize androgens, but convert thecal androgens to estradiol. Also shown are the conversion of progesterone to cortisol as a result of CYP21 and CYP11, which occurs exclusively in the adrenal gland. Irreversible reactions are denoted by a single arrow, and reversible reactions are denoted by double arrows (Gupta & Chia, 2013).

2.1. Estradiol

Estradiol is a female sex hormone produced by the ovaries, adrenal gland and also the placenta during pregnancy. It is the most important hormone during a female's reproductive years, and is required for reproductive and sexual function. In cattle, E2 promotes development of preantral follicles and upregulates steroidogenesis in granulosa and theca cells. It also stimulates follicle growth and development and inhibits granulosa cell apoptosis, and increases the sensitivity of granulosa cells to FSH and LH by promoting the expression of their receptors and regulating the formation of gap junctions between granulosa cells. In cattle, E2 upregulates androgen synthesis in TC and pregnenolone in GC. Shortly before the beginning of deviation between the largest follicle and the second largest follicle, there is a marked difference in concentrations of E2 in the follicular fluid of the two follicles, and a rapid increase in E2 content is a key characteristic of a dominant follicle. In addition, E2 concentrations decrease in subordinate follicles while the dominant follicle continues growing. As the growth of the dominant follicle slows and it starts to regress, estradiol concentration decreases. Thus E2 is a marker for health or atresia of follicles (Beg & Ginther, 2006).

2.2. Progesterone

Progesterone is a steroid hormone involved in pregnancy and embryogenesis and is produced in both TC and GC. During the beginning of the follicular growth there are no differences in P4 levels between the two largest follicles, however some studies have found that after the second largest follicle starts regressing, there is an increase in P4, making unclear the role of progesterone in the process of growth and differentiation. Progesterone secretion by the corpus luteum is required for the success of early pregnancy. Progesterone not only supports endometrial growth but also improves blood flow and oxygen supply by increasing nitric oxide production. It also potentially sustains the survival of the embryo by shifting the immune system towards production of T-helper (Th2) response (Elvin, Yan, & Matzuk, 2000; Shah & Nagarajan, 2013).

3. Mycotoxins

Mycotoxins are natural mold-derived substances of low molecular weight. They are generally produced as secondary metabolites of filamentous fungi and are more common in

hot and humid climates that are more favorable for the growth of molds. Although numerous mycotoxins can be produced by fungi that infect plants, only a few are considered to be toxic for humans and animals. They have adverse effects on humans, animals and crops, and can result in illness and economic losses. Mycotoxin exposure usually happens by ingestion, however there are some other routes such as transdermal and inhalation (Zain, 2011). The term mycotoxin first originated from an outbreak of a mysterious disease in turkeys in southern England in 1962 that was linked to the consumption of peanuts contaminated with secondary metabolites of *Aspergillus flavus* now known as aflatoxins. Fungal products that are toxic to plants are called phytotoxins and the term mycotoxin is used for fungal products that are mainly toxic to vertebrates and other animal groups (Michelle S. Mostrom & Barry J. Jacobsen, 2011). Nowdays, there is a public health concern over the potential for humans to consume animal-derived food products such as meat, milk or eggs that contain residues of mycotoxins or their metabolites. The main socioeconomic impact of mycotoxins includes loss of human and animal life, increased health care costs, reduced animal production, disposal of contaminated foods and feeds, and cost of mycotoxin research (Pfeiffer, Kommer, Dempe, Hildebrand, & Metzler, 2011).

3.1. Classes of mycotoxins

There are many mycotoxins belonging to different classes and which have different mechanisms of action, however the most clinically important ones for human and animal health can be classified into the 5 following groups: alkaloids and indole terpenoids, furanes and coumarines, eicosanoid carboxylic esters, esterogenic and nonsteroidogenic tricothecenes (Yunus et al., 2012). The toxicity of different classes of mycotoxins in various animal species is illustrated in Table 1.

Table 1. Relative toxicity of various classes of mycotoxins on different species. Six major classes of mycotoxins have been evaluated (Mostrom and Jacobsen 2011b).

Toxins	Poultry	Swine	Ruminant
Aflatoxins	+++	++	+
Ochratoxins	+++	+	+
T2 toxins	++	+++	+++
Deoxynivalenol	+	++	++
Zearalenone	+	+++	++
Fumonisin	+	+++	+

(+ = mild toxicity ++ = moderate toxicity +++ = high toxicity)

3.1.1. Alkaloids and indole terpenoids

These mycotoxins are the most prominent class of toxins that are not usually inactivated by rumen microflora. Ergovaline, an ergopeptin, is the most clinically prominent mycotoxin in this group. It comprises proline and two other α-amino acids, linked in an unusual cyclol formation. The metabolism by gastrointestinal microbes or the liver is not required for the immediate physiological effects of ergovaline. The tissues most affected by ergovaline, from greatest to least affected, are the liver, kidney, lung, spleen and skeletal muscles. Fescue toxicosis is the most prevalent intoxication that is linked to tall fescue (*Festuca arundinacea*) consumption (Riet-Correa et al., 2013; Shappell & Smith, 2005).

3.1.2. Furanes and Coumarins

3.1.2.1. Aflatoxins

These mycotoxins are produced primarily by *Aspergillus flavus* and *A. parasiticus* and are identified as B1, B2, G1 and G2. However, Aflatoxin B1 (AFB1) is considered to be the most toxic (Michelle S. Mostrom & Barry J. Jacobsen, 2011). AFB1 requires epoxidation to AFB1 2,3-oxide for activation. Microsomal monooxygenase enzymes biotransform the toxin to the less toxic metabolites aflatoxin M1 (AFM1) and AFQ1. They can be activated to the toxic metabolite with the reactive epoxide group during metabolism mostly in the liver, however, AFB1 is activated in the intestinal tract. Aflatoxin B1 can be converted into aflatoxicol in the rumen, and any AFB1 that escapes rumen degradation is converted into AFM1 by hepatic metabolism and is excreted in milk.

3.1.2.2. Ochratoxins

Ochratoxins are produced by some *Aspergillus* species, mainly *A. ochraceus* and *A. niger*, and some *Penicillium* species. Ochratoxin A is the most prevalent and relevant fungal toxin of this group. Rumen microflora cleave ochratoxin A resulting in the free coumarine named ochratoxin-alpha which is less toxic compared to the parent compound and this may explain the high tolerance of ruminants to ochratoxicosis. Typical signs of ochratoxicosis include renal proximal tubule cell damage and karyomegaly. In ruminants, usually very small amounts of intact ochratoxin A is excreted in milk in contrast to the monogastrics that excrete much of it into milk (M. S. Mostrom & B. J. Jacobsen, 2011; Q. Wu et al., 2011).

3.1.3. Eicosanoid carboxylic esters

Fumonisins are the most prominent mycotoxins in this group. They are produced by *Fusarium verticilloides* which occur mainly in maize, wheat and other cereals. Fumonisin B1 (FB1) is the most prevalent member of this family (Fink-Gremmels, 2008) and is poorly absorbed, with around 6% of orally administered FB1 crossing the GI tract. FB1 is rapidly excreted mostly in its original form, mostly in feces but small amounts are excreted in urine (Fink-Gremmels, 2008). Swine and horses are the species most sensitive to FB1 toxicity.

Although FB1 is resistant to microbial degradation in the rumen, oral bioavailability is lower than the other animals thus severe intoxication does not occur in ruminants (Karlovsky, 1999).

3.1.4. Estrogenic trichothecenes (mycoestrogens)

Zearalenone is a non-steroidal mycotoxin with estrogenic properties, which is produced by fungi belonging to *Fusarium,* mainly *F. graminearum,* in maize, wheat, oats and barley (Buranatragool et al., 2015). The metabolism of ZEA in animals involves the formation of two major metabolites, α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL), which are subsequently conjugated with glucuronic acid. α-ZOL shows higher estrogenic activity than ZEA, but β-ZOL has lower estrogenic effects. Zearalenone exhibits a binding affinity of 10% for estrogen receptor when compared to estradiol-17β, whereas zearalenone possesses a relative binding affinity of 1.8% to that of estradiol-17β. Similar affinities for ZEN have been determined for the estrogen receptor in sheep and calf uterus, these compounds can produce signs of hyperestrogenism (Diekman & Green, 1992). ZEA is metabolized to α- and β-zearalenol (α-ZOL and β-ZOL, respectively) in the rumen by bacteria (Qinghua Wu, Dohnal, Huang, Kuča, & Yuan, 2010) and in numerous tissues by hydroxysteroid dehydrogenase enzymes (Malekinejad, Van Tol, Colenbrander, & Fink-Gremmels, 2006).

Patulin is another lactone derivative mycotoxin that is formed by *Penicillium, Aspergillus* and *Byssochlamus spp*. It is an electrophilic molecule that binds covalently to thiol-containing molecules including glutathione. Glutathione depletion subsequently causes oxidative stress and forms signs of neurotoxicity in animals including tremors and ataxia, recumbency and even death (Fink-Gremmels, 2008; Riet-Correa et al., 2013).

3.1.5. Nonestrogenic trichothecenes

This group consists of deoxynivalenol (DON), nivalenol, T-2 toxin, and diacetoxysripenol, and DON is one of the most prevalent mycotoxins in North America and Europe. These mycotoxins have similar molecular structures and are produced by fungi of *Fusarium* and *Stachybotrys* species usually found growing on grains such as wheat, barley, oats, rye and maize (Zain, 2011).

Deoxynivalenol is a tetracyclic sesquiterpenoid compound and contains seven stereo centers and six oxygen atoms consisting of three alcoholic OH groups, a carbonyl, acyclic ether and an epoxide which would allow for multiple hydrogen bonds (Nagy, Fejer, Berek, Molnar, & Viskolcz, 2005). The epoxide group at position 12-13 is critical for toxicity and allows DON to bind to ribosomes, inducing a reaction known as the ribotoxic stress response (RSR), leading to the activation of various protein kinases such as mitogen-activated protein kinases (MAPKs), the modulation of gene expression, the inhibition of protein synthesis and cell toxicity (Diekman & Green, 1992) (Fink-Gremmels, 2008). Figure 6 illustrates the molecular structure of DON and one of its main metabolites, deepoxy-DON (DOM-1).

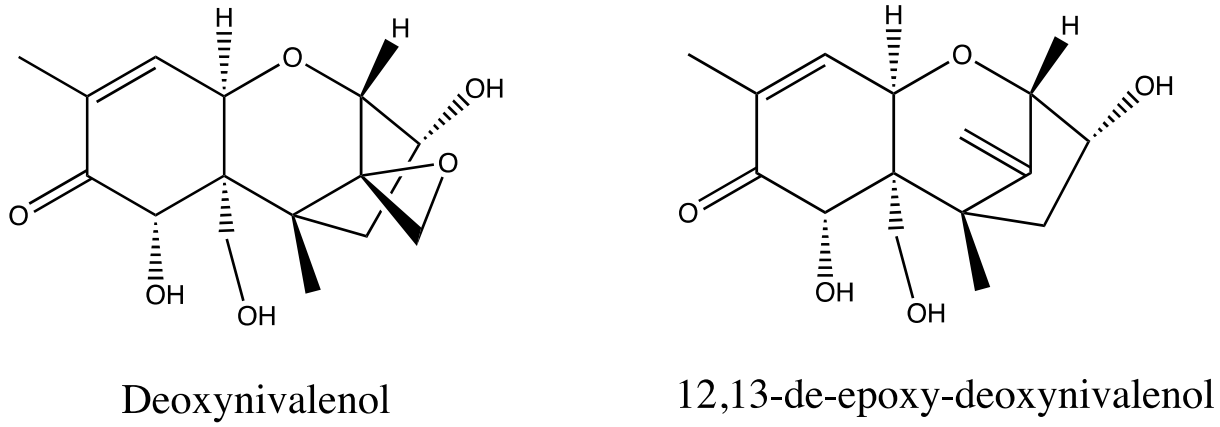

Figure 6. Structure of DON ($C_{15}H_{20}O_6$) and DOM-1($C_{15}H_{20}O_5$). The functional group on these molecules is the epoxide group which allows the molecules to form hydrogen bonds with RNA (Vidal, Sanchis, Ramos, & Marín, 2015).

Deoxynivalenol is absorbed from the upper and lower sections of the gastro-intestinal tract, and can be converted to a number of inactive metabolites. DON can be transformed in the intestine or liver by UDP-glucoronosyltransferase to DON glucoronide conjugate, which is a major excretory product and readily detected in urine (S Dänicke et al., 2005). The molecular mechanism of action of DON is not completely understood, however it results in impairment of immunological functions and elevated serum IgA, and influences both cell-

mediated and humoral immunity. DON decreases feed intake and causes vomiting (hence the common name of vomitoxin). It can also cause mucosal injuries in the stomach and decreases gastric fluid production (Mishra, Dwivedi, Pandey, & Das, 2014). In ruminants, DON is metabolised mainly by the ruminal microflora to de-epoxy-DON (Qinghua Wu et al., 2010), which shows little cytotoxicity compared to DON in vitro (S. Dänicke et al.; Sundstøl Eriksen, Pettersson, & Lundh, 2004).

3.2. Incidence of mycotoxins in Canada and US

DON is the major grain mycotoxin in North America, and is found at concentrations of 3.8-6.5 mg/kg grain, along with ZEA. Daily exposure of Canadians to zearalenone from food consumption has been estimated to be in the range of 0.05-0.10 µg/kg b.w/day in young children, the group with the highest intake on a body-weight basis. The European Union has set maximum safe concentrations of common mycotoxins in foodstuff, and these limits are designed to prevent occurrence of AFM1 toxicosis in humans (Streit et al., 2012; Tittlemier, Gaba, & Chan, 2013).

A study in 2012 on the occurrence of mycotoxins in shipments of Canadian wheat, durum, barely, corn, rye and oats transported from Aug 10, 2010 to July 31, 2012 revealed that DON was the most frequently measured trichothecene, being founds in 231 of 303 samples tested, and at concentrations up to 2.34 mg/kg; the second most frequent mycotoxin was NIV but it was only seen in barely. However the concentrations of DON were markedly correlated to the wheat class and grade - for instance its concentration in durum (0.09 mg/kg) was lower than that for hard red spring (0.21 mg/kg) and the lower grade of wheat has higher median concentrations of DON than higher grades (Tittlemier et al., 2013).

In a study in US during 2008, approximately 70-75% of agricultural commodities used in animal feed were contaminated with various mycotoxin species specially DON and ZEA. Therefore, veterinary practitioners should be aware of potential mycotoxicosis in animal feed and regional mycotoxin problems in grain that could be brought into local channels (Michelle S. Mostrom & Barry J. Jacobsen, 2011).

3.3. Effects of mycotoxins in different species

3.3.1. Pigs

Aflatoxins affect all livestock species, however swine are the most sensitive. Kidney damage, decreased tissue strength, delayed blood clotting as well as decreased resistance to bacterial, fungal, viral and parasitic diseases are attributed to aflatoxicosis. These effects result from aflatoxins interfering with cellular and humoral immune system (Fink-Gremmels, 2008). Zearalenone causes multiple reproductive dysfunctions such as abnormal return to estrus, pseudopregnancy, infertility, stillbirths, neonatal mortality, fetal mummification and abortion in the mature gilt (Buranatragool et al., 2015).

3.3.2. Ruminants

Ruminants are usually considered to be more resistant to the effects of mycotoxins than are monogastric animals since their rumen microflora can metabolize these compounds to less toxic metabolites. However, ruminants can be exposed to diverse toxins by being fed contaminated cereal by-products, poorly preserved silage or baled forage, or even turned to moldy fields in pasture. Due to the negative energy balance during transitional period, cows are considered to be particularly sensitive to the exposure to feed contaminated with moulds, fungal spores and mycotoxins during this time. The main mycotoxicoses observed in grazing cattle include intoxications by indole-diterpenoid mycotoxins, gangrenous ergotism and dysthermic syndrome (hyperthermia) caused by fungus *Festuca arundinacea* (Riet-Correa et al., 2013; Zain, 2011).

3.3.3. Poultry

In the poultry industry, mycotoxins are responsible for high morbidity and mortality especially in young birds, stunted growth, diarrhea, and encephalitis. They cause dermatitis and immunosuppression, which can also make birds prone to several bacterial, viral and parasitic infections leading to huge economic losses. DON is a common *Fusarium* toxin in poultry feed, however chickens are more resistant to the adverse effects of DON compared to the other species. In general, the acute form of mycotoxicosis is very rare in poultry flocks under normal conditions. However, if the diets contain low levels of DON (less than 5 mg/kg

diet) lower productivity, impaired immunity and higher susceptibility to infectious diseases can result (Grenier & Applegate, 2013).

DON has been shown to suppress the antibody response to infectious bronchitis vaccine (IBV) and Newcastle diseases virus (NDV) in broilers (at concentrations of 10 mg/kg feed) and laying hens (at concentrations of 3.5 to 14 mg/kg feed). Moreover, feeding 10 mg/kg of DON decreased tumor necrosis factor alpha (TNF-α) levels in the plasma of broilers (Awad, Ghareeb, Böhm, & Zentek, 2013; Yunus et al., 2012).

3.3.4. Horses

Mycotoxicosis of horses often occurs from fumonisins which is often severe and fatal and can be acute or chronic. The main mycotoxicosis of horses is leukoencephalomalacia caused by fumonisins B1 and B2. They have also been reported as a cause of agalactia and neonatal mortality in mares. Slaframin toxicosis ("slobber") which can cause profuse salivation in horses results from fumonisin intoxication. Overall, horses appear to have a relatively low prevalence of reported mycotoxicosis among domestic animals but they are extremely sensitive to the fumonisins (Osweiler, 2001; Riet-Correa et al., 2013).

3.4. Developmental toxicity

Ochratoxin A (OTA) causes congenital defects such as increased embryo resorption, dead fetuses, reduced birth weight and craniofacial abnormalities in humans, rats, mice, hamsters, quail and chickens. It has also teratogenic effects and can alter sperm quality in human (Malir, Ostry, Pfohl-Leszkowicz, & Novotna, 2013). In pregnant rats, early gestational exposure to ZEN causes maternal toxicity and reproductive disfunction that can lead to early embryonic death and delayed fetal development (Y. Zhang et al., 2014). Intoxication with fumonisin B1 disturbs the expression of genes encoding enzymes of sphingolipid metabolism in the maternal liver, uterus, fetus and placenta (Liao et al., 2014). Exposure to aflatoxin B1 during prenatal development in rat caused gene mutations in G:C (Guanine-to-Cytosine) to T:A (thymine-to-Adenine) in both mother and offspring (Chawanthayatham et al., 2015).

3.5. Gastrointestinal toxicity

Treating undifferentiated and differentiated Caco-2 intestinal cells with aflatoxins B1 and M1 at various concentrations for 72 h increased LDH release and reactive oxygen species (ROS) levels, and caused damage to the DNA of these intestinal cells. However, AFB1 was found to be more toxic than AFM1 for Caco-2 cells (J. Zhang et al., 2015). Fumonisin B1 can induce lipid peroxidation leading to increased malondialdehyde levels and a subsequent increase in membrane permeability and ROS activity (Garbetta et al., 2015).

In an in vitro study, α-ZOL and β-ZOL both were toxic for intestinal porcine epithelial cells (IPEC-1) at concentrations of 10-100 μM. However, at doses lower than 10 μM, only β-ZOL demonstrated significant toxicity for IPEC-1 cells (Marin, Motiu, & Taranu, 2015). DON and type B trichothecenes induced necrosis of intestinal epithelium through alterations in cell morphology and differentiation and also in the barrier function of this epithelium in human and animals. Addition of DON significantly decreased the relative density (weight:length) of the small intestine by decreasing the height of the villi (Yunus et al., 2012).

3.6. Effect of mycotoxins on the immune system

Trichothecenes have both immunostimulatory and immunosuppressive effects depending on dose, frequency, exposure time and type of immune function assay. Exposure to low concentration levels usually upregulate gene expression of cytokines and chemokines, and concurrently stimulates immune response, whereas in high concentrations they promote apoptosis of leukocytes with concurrent immune suppression (J. J. Pestka, 2008).

Ochratoxin A is an immunosuppressant compound which reduces the size of vital immune organs and depresses the antibody response by inducing cell apoptosis and necrosis and inhibiting protein synthesis. Type B trichothecenes can affect cytokine production by interfering with the cross-talk between intestinal epithelium cells and intestinal immune cells. DON can also target immune cells and activates the ribotoxic stress response (RSR) involving MAPK pathway activation (Al-Anati & Petzinger, 2006; Pinton & Oswald, 2014).

3.7. Effect of mycotoxins on the reproductive system

In swine, 22 mg/kg of ZON can markedly alter the function of the reproductive system and affect follicular and embryo development. However, DON affects the reproductive system of pigs more seriously via indirect effects such as reduced feed intake, reduced growth and impairment of function of vital organs such as the liver and spleen (Malir et al., 2013).

Fumonisin B1 increased P4 production from porcine granulosa cells, and had no effect on E2 production, and inhibited granulosa cell proliferation. Addition of α-zearalenol increased P4 production, and acted in synergy with FB1 to further increase P4 production and to stimulate granulosa cell numbers (Cortinovis, Caloni, Schreiber, & Spicer, 2014). Neither FB1 nor α-zearalenol alone altered E2 production, however, in combination they decreased E2 production (Cortinovis et al., 2014).

In an *in vitro* study, different doses of DON (0.94, 1.88, 3.75 and 7.5 μM) were tested to identify the effect of this mycotoxin on oocyte maturation in pigs. At a concentration of 1.88 μM (556.2 ng/ml), DON significantly decreased oocyte maturation in cumulus-oocyte complexes (COCs). However, higher concentrations of 7.5 μM had no effects on embryonic development (Alm, Greising, Brussow, Torner, & Tiemann, 2002). Both *in vivo* and *in vitro* exposure to DON decreased oocyte and embryo development and inhibited the uterus cell proliferation and modulated the process of translation in pig (Tiemann & Danicke, 2007).

In pigs, DON inhibited the secretion of IGF-I from GC in vitro, whereas P4 release and the expression of cyclin B1 (a proliferative marker) was increased by DON (at 1000 ng/mL but not at 10 and 100 ng/mL). DON stimulated PCNA (a marker of proliferation) expression at 100 and 1000 ng/mL but not at 10 ng/mL. At all doses DON did not affect the expression of caspase-3, which is a marker of apoptosis (Medvedova et al., 2011). These data suggest that DON does not negatively impact granulosa cells in pigs.

Recent data from studies with bovine granulosa cells have shown that DON inhibited estradiol and progesterone secretion and reduced the abundance of CYP19A1 mRNA, although had no effect on genes encoding progestagenic proteins (CYP11A1 and STAR) (Guerrero-Netro, Chorfi, & Price, 2015). Treatment of granulosa cells with DON induced cell apoptosis, increased FASLG mRNA levels, caused rapid and transient increase in

phosphorylation of MAPK3/1, and prolonged phosphorylation of MAPK14 (p38) and MAPK8 (JNK) in granulosa cells.

Exposure of bovine granulosa cells to β-Zol at 31 μM and α-Zol at 3.1 μM alone and combined with DON at 3.3 μM negatively affected cell proliferation. DON and α-Zol decreased steroid production while β-Zol at high concentration had stimulatory effects. DON, alone and combined with α-Zol and β-Zol stimulated CYP11A1 mRNA abundance, whereas β-Zol alone inhibited CYP11A1 mRNA levels (Pizzo, Caloni, Schreiber, Cortinovis, & Spicer, 2016; Pizzo, Caloni, Schutz, Totty, & Spicer, 2015). Data from our laboratory show that DOM-1 also increases the rate of apoptosis in theca cells in vivo (Guerrero-Netro et al., unpublished), although the pathway through which this is achieved is unknown.Mechanism of toxicity of DON

4. Mechanism of toxicity of DON

4.1. The ribotoxic stress response

The RSR is a process by which translational inhibitors and other translation-interfering toxicants, termed ribotoxins, bind to ribosomes and rapidly activate mitogen-activated protein kinases. The activation of RSR by toxicants disturbs the function of the 3′-end of the large 28S ribosomal RNA. During protein synthesis, this region of the ribosome is involved in aminoacyl-tRNA binding, peptidyltransferase activity, and ribosomal translocation. Depending on the cell type, toxicant-induced disruption of this activity results in activation of Jun N-terminal kinase (JNK) and protein kinase p38 (p38) and/or alterations in extracellular regulating kinase 1/2 (ERK1/2) signaling. In most cases, active ribosomes appear to be required as mediators of this signaling response and many of the inducers of the ribotoxic stress response at least partially inhibit protein synthesis (Laskin, Heck, & Laskin, 2002).

Trichothecenes such as T2-triol, nivalenol, scirpentriol and DON are potent ribotoxins and trigger immunosuppression, apoptosis and rRNA cleavage at least in part through the RSR (Moon & Pestka, 2002). The process through which DON triggers RSR has been studied in different cell types (Figure 7), and 4 different ways to inhibit protein synthesis have been reported: 1) DON interferes with the peptidyl transferase function on the ribosome; 2) DON causes degradation of 18S and 28S rRNA through apoptotic pathways; 3) DON induces

activation of protein kinase R (PKR) which phosphorylates eukaryotic initiation factor 2 α-subunit (eIF2α); and 4) DON up-regulates microRNAs, which potentially target mRNAs for translation inhibition, especially ribosomal proteins (He, Zhou et al. 2012)(He, Zhou, Young, Boland, & Scott, 2010). Studies in Jurkat T cells have shown that DON, NIV and T-2 have the ability to up-regulate p38 and JNK activation in different ways, indicating that structural differences between trichothecenes affect the form of RSR they trigger, and that increased JNK/p38 activation is associated with increased activation of caspase-3 and apoptosis (Shifrin & Anderson, 1999).

Trichothecenes can also increase the activity of prostaglandin-endoperoxide synthase 2 (PTGS2) and lead to apoptosis. In murine macrophages, treatment with DON increased PTGS2 mRNA and protein levels, prostaglandin secretion and phosphorylation of ERK1/2, p38 and JNK. Addition of p38 and ERK 1/2 inhibitors resulted in suppression of PTGS2 levels, suggesting a possible way for DON to upregulate pro-inflammatory and apoptotic genes (Moon & Pestka, 2002). Another possible mediator of PTGS2 up-regulation is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a protein that controls transcription of DNA in stressed cells and regulates the immune response. Knocking down NF-kB in human and rat arterial smooth muscle cells reduced ERK1/2 phosphorylation and reduced PTGS2 expression, suggesting that NF-kB might be a potential regulator of RSR (Woywodt et al., 2001; Z. Yan et al., 2000).

In macrophages, MAPK activation after DON exposure seems to be dependent upon the activation of c-terminal Src kinase, as pharmacological inhibition of Src kinase activity reduced DON-induced phosphorylation of P38 and JNK, and decreased the expression of hematopoietic cell kinase (HCK), part of the Src family. Further, treatment with DON increased expression of HCK, strongly implicating this kinase in the mycotoxin-induced RSR (Zhou, Neale, Pomper, & Kozikowski, 2005).

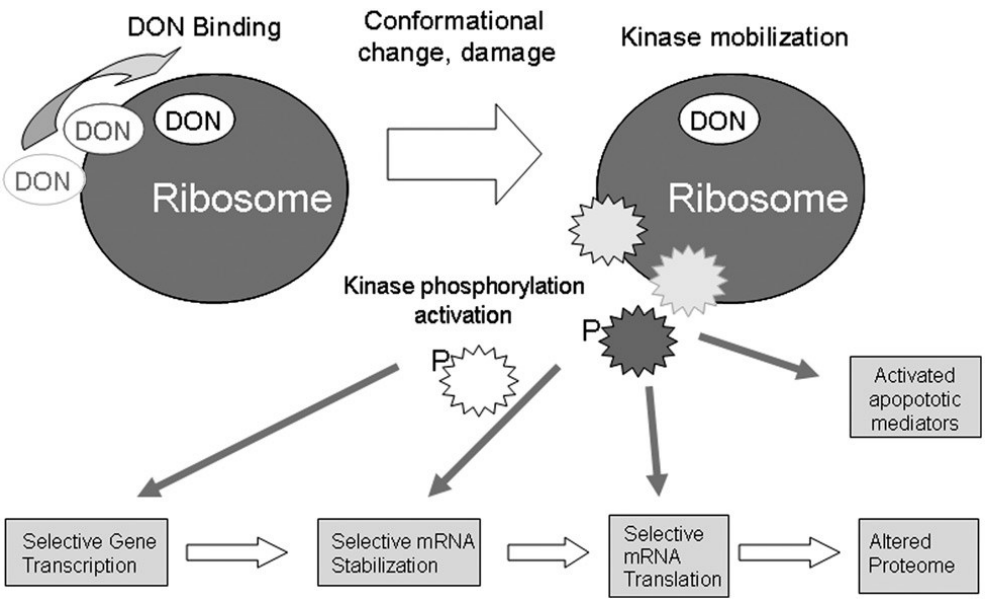

Figure 7. DON-induced ribotoxic stress response mechanism. In response to the ribosome damage caused by DON, signaling pathways including MAPK and PI3K/AKT are activated that lead to the phosphorylation or dephosphorylation of these molecules. These molecules mediate key biological processes that regulate different aspects of cell function such as cell proliferation or apoptosis (James J Pestka, 2008).

4.2. Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases are key signal-transducing enzymes that are activated by a wide range of extracellular stimuli. They are responsible for the induction of a number of cellular responses, such as changes in gene expression, proliferation, differentiation, cell cycle arrest and apoptosis. MAPKs are generally constitutively expressed and catalytically inactive in their base form. In order to become active, they require multiple phosphorylation events in their activation loops. This tandem activation loop phosphorylation is performed by members of the Ste7 protein kinase family, also known as MAP2 kinases. MAP2 kinases in turn, are also activated by phosphorylation induced by a number of different upstream serine-threonine kinases (MAP3 kinases). These classical pathways can effectively convey stimuli from the cell membrane (where many MAP3Ks are activated) to the nucleus (where only MAPKs may enter) or to many other subcellular targets (Theodosiou & Ashworth, 2002).

It is well known that the expression of specific genes can occur as a consequence of MAPK activation. A notable example is the transcriptional activation of the immediate early genes c-fos (FOS) and c-jun (JUN). Some toxins such as verotoxin (Shiga toxin) effectively induce MAPK phosphatase 1, a dual-specificity MAPK phosphatase. This enzyme can inactivate MAPK3/1 as well as MAPK8 and MAPK14 and can act in a negative feedback loop, balancing the increase in activity of MAPK and potentially suppressing the responses of cells to ribotoxic stressors. This type of complex feedback may be important in preventing toxicity and/or initiating repair (Enslen & Davis, 2001; Rolli-Derkinderen & Gaestel, 2000).

Figure 8 illustrates the cascade of Ras/Raf/MEK/MAPK signaling pathway that leads to the activation of some transcription factors involved in various cell function. Ras is a small GTP-binding protein, which is the common upstream molecule of several signaling pathways including Raf/MEK/MAPK, PI3K/AKT and RalEGF/Ral. Different mutation frequencies have been observed between Ras genes in human cancer (Ki-Ras>Ha-Ras), and these Ras proteins show varying abilities to activate the Raf/MEK/MAPK and PI3K/AKT cascades. Ki-Ras has been associated with Raf/MEK/MAPK while Ha-Ras is associated with PI3K/AKT activation. Raf is a serine/threonine (S/T) kinase and is normally activated by a complex series of events including recruitment to the plasma membrane mediated by an interaction with Ras, dimerization of Raf proteins, and phosphorylation on different domains. Moreover, Raf activity is modulated by adaptor proteins, including Bag1, Hsp70 and 14-3-3. Inhibition of Ras activity blocks activation of Raf molecule and the downstream MEK activity (Chang et al., 2003; Tzivion, Shen, & Zhu, 2001).

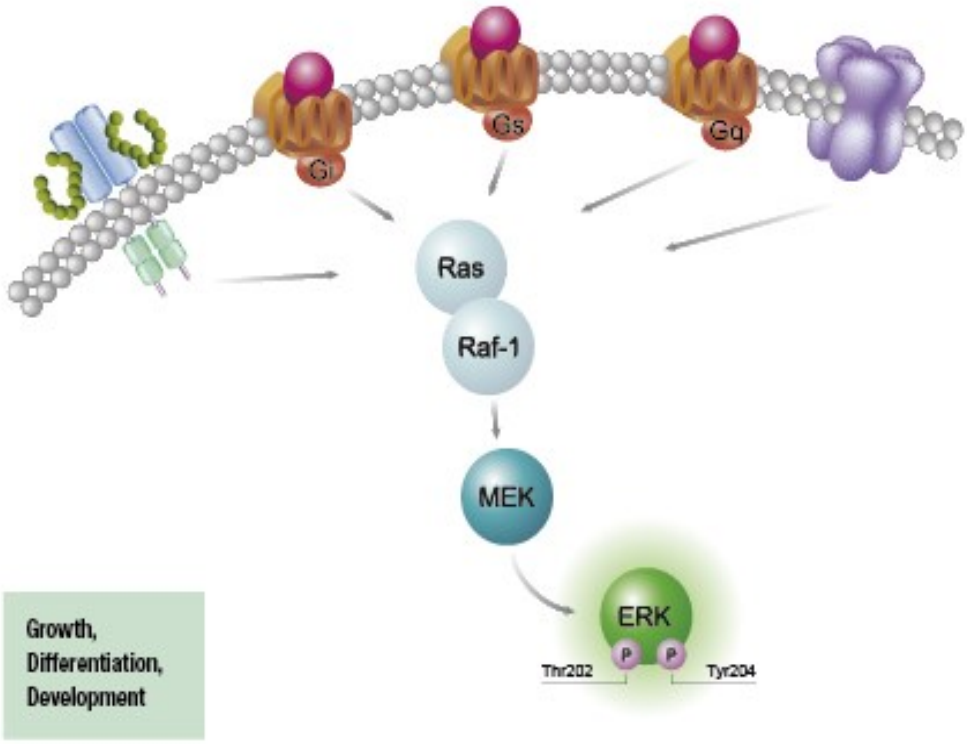

Figure 8. MAPK signaling pathways. A highly conserved signal-transduction pathway is activated in which the signal induced by ligand binding is transfered via GRB2 to Ras, leading to its activation. Activated Ras then induces a kinase cascade that culminates in activation of MAPK family members such as MAPK3/1. This serine/threonine kinase translocates into the nucleus, phosphorylates many different proteins including transcription factors that regulate expression of important cell-cycle and differentiation-specific proteins (Mallia & Vassallo, 2015).

5. Mass spectrometry

5.1. MS-based protein identification strategies

There are various approaches for protein analysis in mass spectrometry (MS), however, “top-down” and “bottom-up” are the main approaches. In top-down MS, intact proteins are injected into the gas phase and are fragmented, providing information on the masses of proteins and fragments thereof. This approach provides the most complete profile of proteins

within the sample. However, in bottom-up MS the proteins are first enzymatically or chemically digested to create peptide fragments that are then injected into the gas phase. These smaller peptides are much easier to analyze with low resolution MS and thus this is currently the most popular MS approach. Figure 9 shows the difference between these two major approaches (Chait, 2006).

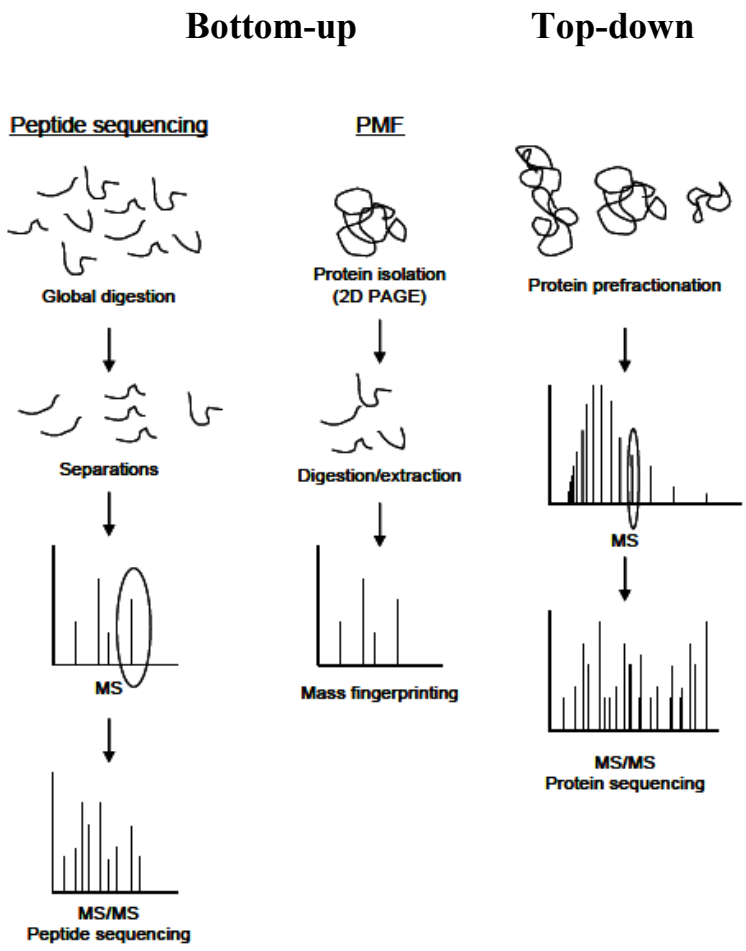

Figure 9. Strategies for analysis of MS-based proteomics. In the "bottom-up" strategy, proteins are chemically digested using proteolytic enzymes such as trypsin, then the identity of the proteins are detected using techniques such as "peptide sequencing" or "peptide mass mapping". In the "top-down" strategy the intact proteins are directly analyzed by MS, which requires high performance MS. It also interprets very complex tandem MS spectra (Chait, 2006).

5.2. Bottom-up proteomics

"Bottom-up" proteomics is a popular technique to identify proteins and their amino acid sequences, and was the approach used in this study. In "Bottom-up" MS, the proteins may be first purified by gel electrophoresis and then enzymatically digested in gel to create relatively few tryptic fragments. As an alternative, entire samples can be digested and the resulting peptides separated by liquid chromatography before mass spectrometry (MS). By comparing the masses of the proteolytic peptides or their tandem mass spectra with the predicted mass, peptides can be detected and subsequently the original proteins can be identified using combination of multiple peptide recognition (Figure 10) (Chait, 2006; Wolters, Washburn, & Yates, 2001).

The bottom-up proteomics needs higher sensitivity spectrometers and thus there will be better separation of peptides compared with the top-down approach. However, identifying a protein depends on correctly matching to peptide fragments and cases arise in which a fragment may match to multiple proteins or none at all. In this regard, a "middle-down" proteomics, which is a combination of bottom up and top down approach, has become popular, moreover, it can detect large protein fragments and avoids the redundant peptide sequences (Y. Zhang, Fonslow, Shan, Baek, & Yates III, 2013).

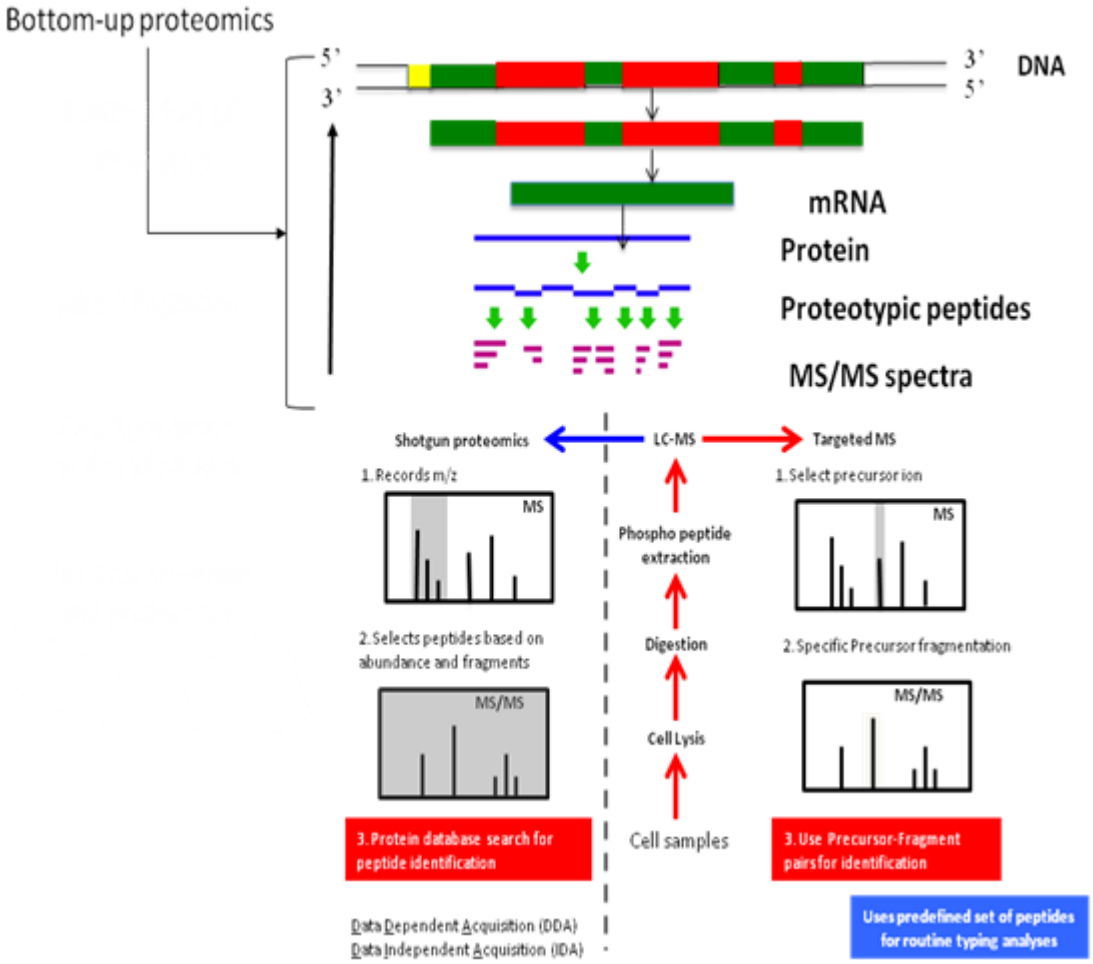

Figure 10. Bottom-up proteomics. LC/MS spectra to identify an unknown protein using Shotgun proteomics or targeted MS approach. In this technique phosphopeptides are first extracted from digestion of lytic cells or from applying various proteomic approaches. In shotgun proteomics these phosphopeptides are separated in terms of mass to charge and then according to the abundance of the fragment peptides the proteins are identified. In targeted MS, the specific fragments from the precursor ions are used and the predefined set of peptides are applied to identify protein molecules. (Chait, 2006).

The principal analytical methods used in bottom-up analysis of peptide fragments to identify proteins are, 1) peptide mass fingerprinting (PMF) and 2) peptide sequencing that use tandem mass spectrometry (MS/MS) (Chait, 2006).

5.2.1. Peptide mass fingerprinting

In PMF, a set of peptides which have predictable masses are produced following enzymatic digestion by trypsin which is an endoprotease. Trypsin cleaves the amide bond on the carboxyl side of lysine and arginine residues. The trypsin ensures the presence of at least one basic amino acid residue into the fragment peptides, and that there will be a location for positive ionization through protonation. As the locations of the enzymatic digestion are predictable, the masses of the peptides, resulting from the digestion of a given protein can be calculated. The list of peptide masses is unique for a particular protein, and can be used to identify the protein if its amino acid sequence is known. However, the PMF technique has some limitations, for instance, the protein sample must be fairly simple, otherwise, the tryptic peptides from contaminating proteins would lead to inaccurate identification of the proteins. Thus, the adequate separation of the sample through one or two dimensional gel electrophoresis is required. There can also be some unknown PTMs (post-translational modifications) that result in the miscalculation of the expected peptide masses. Nevertheless, in "peptide sequencing" there is no need for protein purification to identify peptides by tandem mass spectrometry (Xie, Liu, Qian, Petyuk, & Smith, 2011).

5.2.2. Peptide sequencing

In "peptide sequencing", the detected peptides in the mass spectrometer are first isolated and then fragmented, using collision-induced dissociation (CID). In CID, the energy causes the increase in collisions of atoms within a collision chamber. In peptide CID, MS/MS of ions with positive charge is created from cleavage of the amide bond along the peptide fragments. The naming convention for these ions is based on the "Roepstorff and Fohlman" technique in which the "b" ions are the fragment containing the N-terminus, and "y" ions are the fragment peptides containing the C-terminus. Subsequently the amino acid sequence of the peptides can be detected by calculation of the observed differences in mass between ionized fragments and their parent mass. Eventually, the identity of the proteins can be detected based on their amino acid sequence of the fragments. Figure 11 illustrates the location of various ionized fragments along a peptide following CIDs (Linscheid, Ahrends, Pieper, & Kühn, 2009) (Roepstorff & Fohlman, 1984).

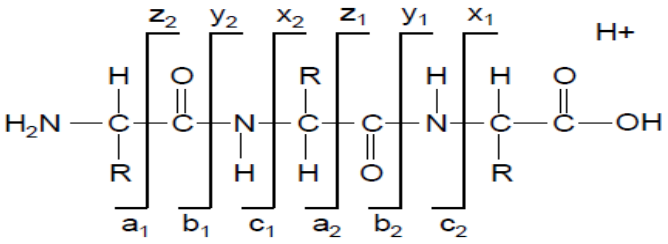

Figure 11. Main fragmentation pathways of peptides in collision induce dissociation mass spectrometry. This convention is based on the "Roepstorff and Fohlman" annotation in which the black lines indicate the location of fragmentation along the peptide. Peptide fragments with a charge on the N-terminal side are considered to be either a, b, or c ions, and the fragments with a charge on the C-terminal side are named z, y, and z ions. This fragmentation mostly produces b and y ions along the peptide fragments, and generate fragments with masses correlated to the mass of their amino acid residues (Linscheid et al., 2009; Roepstorff & Fohlman, 1984).

5.3. Quantitative liquid chromatography-mass spectrometry (LC-MS)

The proteomic approaches described above are useful for identifying the proteins in a sample, and are largely qualitative. Quantitative LC-MS, however, offers the ability to identify proteins and also quantify the abundance of target proteins within a sample. The relative quantification of peptides generally involves either "label-free" or "stable isotope labeling" techniques to distinguish the differences in protein abundance between samples, and the results are usually represented as fold changes. Altogether, the label-free approaches are more dynamic and have broader proteome spectra, whereas stable isotope labeling approaches are more precise and accurate to quantify protein samples. "Absolute quantification" is a common approach which quantifies the exact concentration of a peptide or protein in a sample and requires the use of an appropriate internal standard (Xie et al., 2011).

The peptide sequence can be derived from the MS/MS spectrum due to the sensitivity of the fragment peptides to the CIDs in the MS/MS analysis in which the *b*- or *y*-type ions (N- or C-terminal fragments, respectively) are generally produced. Some post-translational modifications can also be detected by dynamic modification on certain amino acid residues (e.g. Ser, Thr, or Tyr) in the database search. Each peptide can also be quantified at the extracted ion chromatogram level. Figure 12 illustrates the different steps involved in LC-MS-based proteomic approach (Ong & Mann, 2005; Wilm, 2009).

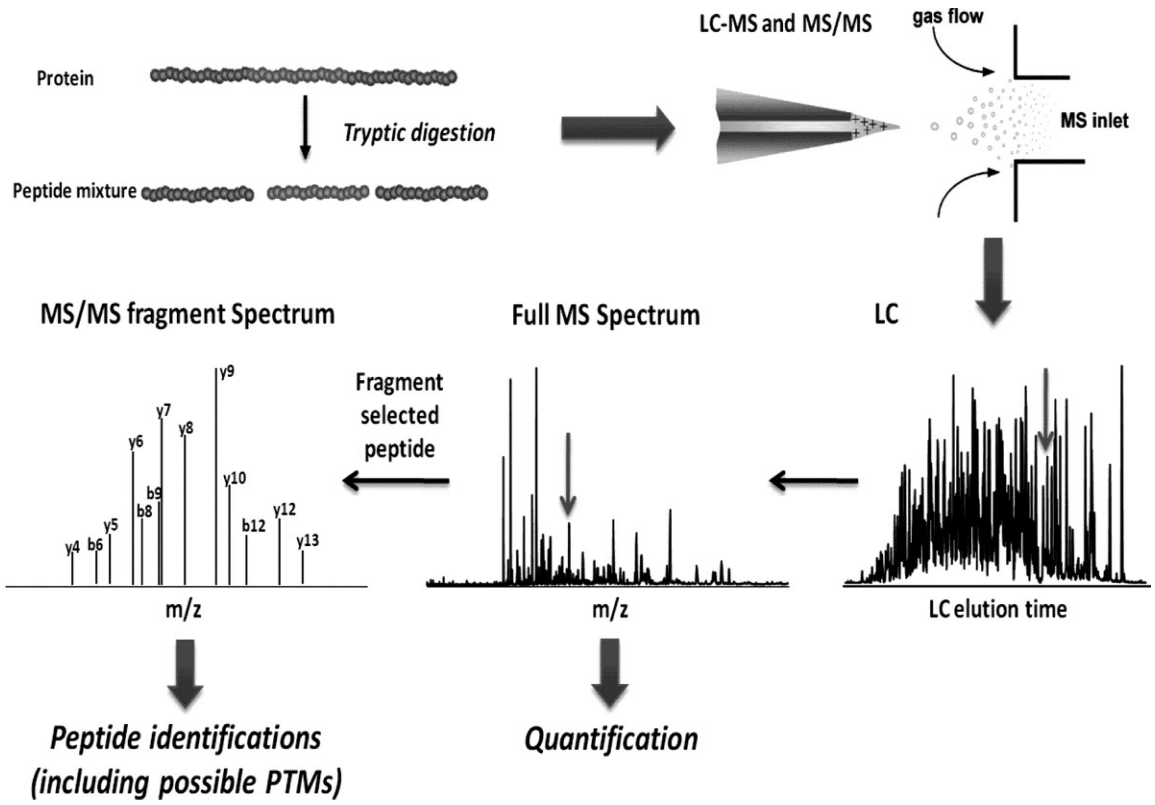

Figure 12. LC-MS-based global proteomics to identify and quantify fragment peptides.

Peptides extracted following tryptic digestion are separated by LC and ionized by electrospray before entering the mass spectrometer. A full MS spectrum is obtained for the peptides that are eluting from the LC column at any given time; one of the most intensive peptide ion is then isolated and fragmented to obtain the MS pattern of its fragments. Most of the CIDs produce predominantly b- or y-type ions and the peptide sequence can be identified from MS/MS spectrum. The possible PTMs can also be detected on certain amino acid residues (e.g. ser, Thr, or Tyr) and the quantification is performed at the extracted ion chromatogram or MS/MS spectrum level (Xie et al., 2011).

Chapter 2: Hypothesis and objectives

Hypothesis

From the literature review, it is clear that mycotoxins have a negative impact on granulosa cell function in ruminants. There are relatively few studies of DON and DOM-1 in cattle, but the data available suggest that both compounds also alter theca cell function, although the mechanism of action remains unknown.

The hypothesis of this study is that DON and DOM-1 alter the phosphorylation status of key intracellular signaling molecules in ovarian theca cells, including MAPK family members such as MAPK3/1, MAPK13 and MAPK14.

Objective

The objective of this study was to identify phosphorylated proteins in theca cells in response to DON and DOM-1 by mass spectrometry.

Chapter 3: Materials and methods

Cell culture

All materials were obtained from Life Technologies Inc. (Thermo Fisher Scientific, Burlington, ON, Canada) unless otherwise stated. Bovine theca cells were cultured in serum-free conditions that maintain testosterone and progesterone secretion and responsiveness to LH (Glister, Richards, & Knight, 2005). Bovine ovaries were obtained from adult cows, independently of the stage of the estrous cycle at the slaughterhouse and transported to the laboratory at 30 ºC in phosphate-buffered saline (PBS) containing penicillin (100 IU) and streptomycin (100 µg/mL). Follicles (4–6 mm diameter) were bisected within the ovarian stroma, gently scraped to remove granulosa cells, and the theca 'shells' were peeled from the stroma with forceps. Pooled theca layers were incubated with collagenase (type IV, 1 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) and trypsin inhibitor (100 ng/mL; Sigma) in a water bath at 37 ºC for 45 min with agitation every 10 min. The resulting supernatant was filtered through a 150 mesh steel sieve (Sigma-Aldrich), centrifuged (800 g for 10 min) and the pellet resuspended in PBS before being subjected to an osmotic shock treatment to remove red blood cells. After washing, cells were resuspended in culture medium McCoy's 5A modified medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 µg/mL fungizone, 10 ng/mL bovine insulin, 2 mM L-glutamine, 10 mM HEPES, 5 µg/mL apotransferrin, 5 ng/Ml sodium selenite, and 0.1 % BSA (all purchased from Sigma-Aldrich) and LH. Cell viability was assessed by Trypan blue dye exclusion, seeded into 24-well tissue plates (Sarstedt Inc., Newton, NC, USA) at a density of 500,000 viable cells in 1 mL, and cultured at 37 ºC in 5 % CO_2, 95 % air for up to 6 days with medium changes every 2 days.

Experimental treatments in vitro

Certified Biopure Standard grade DON and DOM-1 in acetonitrile were purchased from Romer Labs (Tulln, Austria), and were reconstituted in methanol for cell culture studies. To assess the effect of DON and of DOM-1 on intracellular pathway activation, cells were

treated on day 5 of culture with 1 ng/mL DON or DOM-1 for 30 minutes, and cells were recovered in RIPA buffer to measure the phosphorylation status of key protein kinases. Control cells were not exposed to DON or DOM-1. All experiments were performed with three different pools of cells each collected on a different occasion.

Protein extraction

Proteins (50 µg) were isolated by precipitation with 50 µL of ethanol, centrifugation at 9,000 g for 10 min, and the protein pellet was dried for 20 min in a vacuum centrifuge set at 60 ˚C. The protein pellet was dissolved in 50 µL of 100 mM ammonium bicarbonate (pH 8.5) and the solution was sonicated for 30 min at maximum intensity to improve protein dissolution. The proteins were denatured by heating at 120 ˚C for 10 min, cooled for 15 min at room temperature, and proteins were reduced with 20 mM DTT at 60 ˚C for 60 min. Then proteins were alkylated with 40 mM IAA at room temperature for 30 min. One µg of proteomic-grade trypsin (i.e. ratio 1:50) was added and the mixture incubated at 40 ˚C for 24 h. The protein digestion was quenched by adding 50 µL of a 1% TFA solution, followed by centrifugation at 9,000 g for 10 min, and the supernatants were transferred into injection vials for analysis.

Phosphopeptide extraction

Phosphopeptide enrichment was performed with the Titansphere ™Phos-Tio Kit, which is based on titanium dioxide (TiO_2) enrichment. After equilibration of the TiO_2 matrix by sequencial washing with Buffer A (2% trifluoroacetic acid in acetonitrile solution,1:4 vol:vol) and Buffer B (provided in the kit), peptide sample (15 µL) was diluted in 50 µL Buffer B and centrifuged through the TiO_2 three times at 1000 g for 10 min to adsorb the phosphopeptides to the matrix. Non-phosphorylated peptides were washed off the matrix by sequential washes with Buffer B and Buffer A, and phosphopeptides were eluted 5% ammonium hydroxide followed by 5% pyrrolidine solution

.

Mass spectrometry

A Thermo Scientific Q-Exactive Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced with a Thermo Scientific UltiMate 3000 Rapid Separation UHPLC system using a pneumatic assisted heated electrospray ion source. The chromatography was achieved using a gradient mobile phase along with a C8 column (Thermo Biobasic 100 × 1 mm) with a particle size of 5 μm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 1 min, the ratio was maintained at 5:95. From 2 to 62 min, a linear gradient was applied up to a ratio of 50:50 and maintained for 3 min. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 15 minutes for a total run time of 80 minutes. The flow rate was fixed at 75 μL/min and 2 μL of samples were injected.

MS detection was performed in positive ion mode and operating in scan mode at high-resolution, and accurate-mass (HRAM). Nitrogen was used for sheath and auxiliary gases and they were set at 10 and 5 arbitrary units. The heated ESI probe was set to 4000 V and the ion transfer tube temperature was set to 300°C. The default scan range was set to m/z 400-1500. Data was acquired at a resolving power of 140,000 FWHM (or full width at half maximum) using automatic gain control target of $3.0x10^6$ and maximum ion injection time of 200 msec. Data independent analysis (DIA) was performed using a survey scan (m/z 600-1200) along with 12 targeted MS^2 (tMS^2) scan events with an isolation window of 50 Da were performed using a loop count of 6 events. Targeted product ion spectra were acquired a resolving power of 17,500 (FWHM), using automatic gain control target of $1.0x10^6$ and maximum ion injection time of 100 msec. The collision energy was set to 25 and the isolation window was set to 1.5 Da. All samples were analyzed in full-scan MS using a resolving power of 140,000 (FWHM) and in DIA mode.

Bioinformatic analyses

Database searching was performed on Proteome Discoverer software (version 1.4) with Uniprot bovine protein database (extracted FASTA file). Mass tolerance of precursor and fragment were set at 5 ppm and 10 ppm, respectively. Phosphorylation at Y and T amino acids was set as a variable post translational modification. Quantification was based on MS^1 ion

intensity and peptide identification was based on precursor ion (MS^1) and at least three characteristic (MS^2).

Data from all experimental groups were analyzed using SIEVE (Thermo Scientific, San Jose Ca), a label-free differential expression software that aligns the MS spectra over time from different data sets and then determines structures in the data (m/z and retention time pairs) that differ. The following parameters were set to align the retention time and generate the frames needed for abundance calculations. Alignment Parameters; Alignment Bypass = False, Correlation Bin Width = 1, RT Limits For Alignment = True, Tile size = 300, Max RT Shift = 0.2, m/z Min = 400, m/z Max = 1,500, Frame time Width (min) = 2.5 min, Frame m/z width = 10 ppm, Retention Time Start = 2.0 min, Retention Time Stop = 65 min, Peak Intensity threshold = 100,000. Significance was calculated within SIEVE using a standard t-test and results were filtered using the identification criteria stated above. Statistical significance was set at a p value < 0.05.

Identification of gene ontology (GO) annotation terms and analysis of networks between differentially phosphorylated proteins was performed by Reactome (Fabregat et al., 2016), and illustrated by STRING protein interaction software (Szklarczyk et al., 2014).

Chapter 4: Results

DON upregulated and downregulated phosphorylation of 93 and 255 peptides respectively

A volcano plot of 2273 phosphopeptides detected after treatment with DON is presented in Figure 13A. A total of 93 peptides were phosphorylated and 254 peptides were dephosphorylated in response to DON compared with non-treated control cells. Of the phosphorylated peptides, 25 were identified as known proteins, and of the dephosphorylated peptides, 21 peptides were identified as known proteins as illustrated in Figure 13B. In this graph the values greater than +1 and lower than -1 represent more than 2-fold increase or decrease in phosphorylation respectively.

DOM-1 increased and decreased abundance of 19 and 20 known peptides respectively

DOM-1 altered the abundance of 322 phosphorylated peptides compared with non-treated control cells, as illustrated in Figure 14A. Of the phosphopeptides with increased abundance, 19 were from known proteins. Of the phosphorylated peptides with lower abundance after DOM-1 treatment, 20 were identified as known proteins; these are illustrated in Figure 14B.

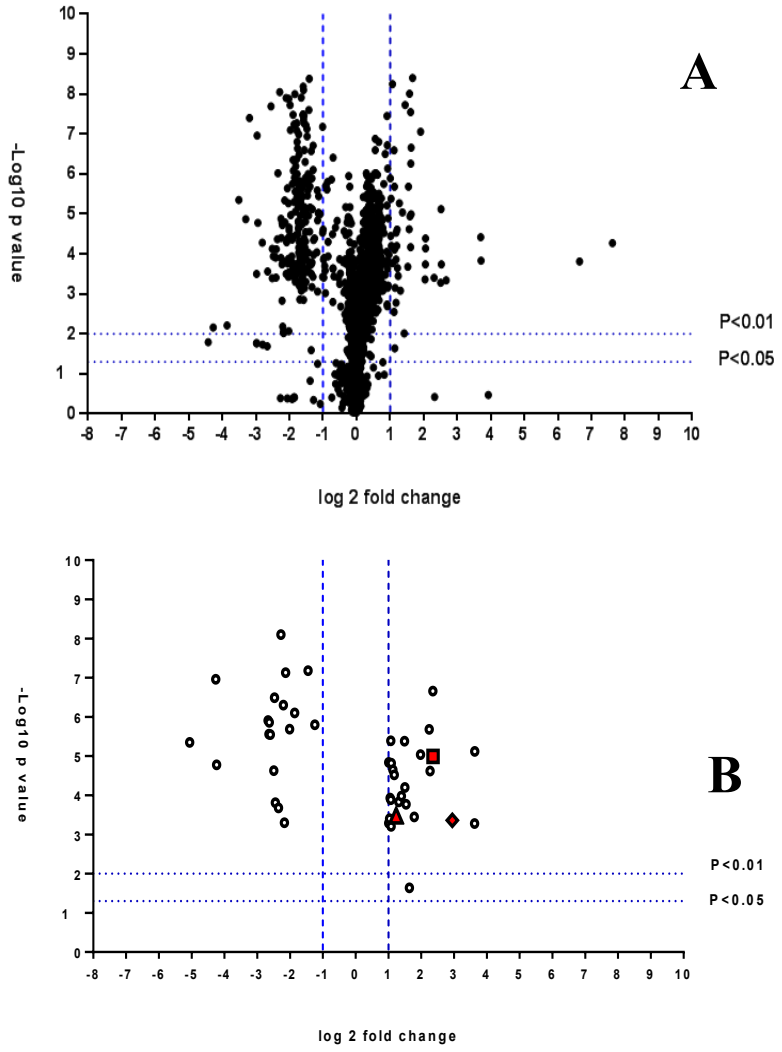

Figure 13. A volcano graph illustrating distribution of different upregulated and downregulated fragment peptides in bovine theca cells exposed to DON. The log 2 fold values greater than 1 and lower than -1 represent more than 2 fold increases or decreases in phosphorylation, respectively. The Y axes indicate significance levels. Graph A illustrates all the phosphorylated proteins inside the cells. Identified proteins with significantly increased and decreased phosphorylation status are illustrated in graph B. The core MAPKs 1, 13 and 14 are shown as red triangles, diamonds and squares, respectively.

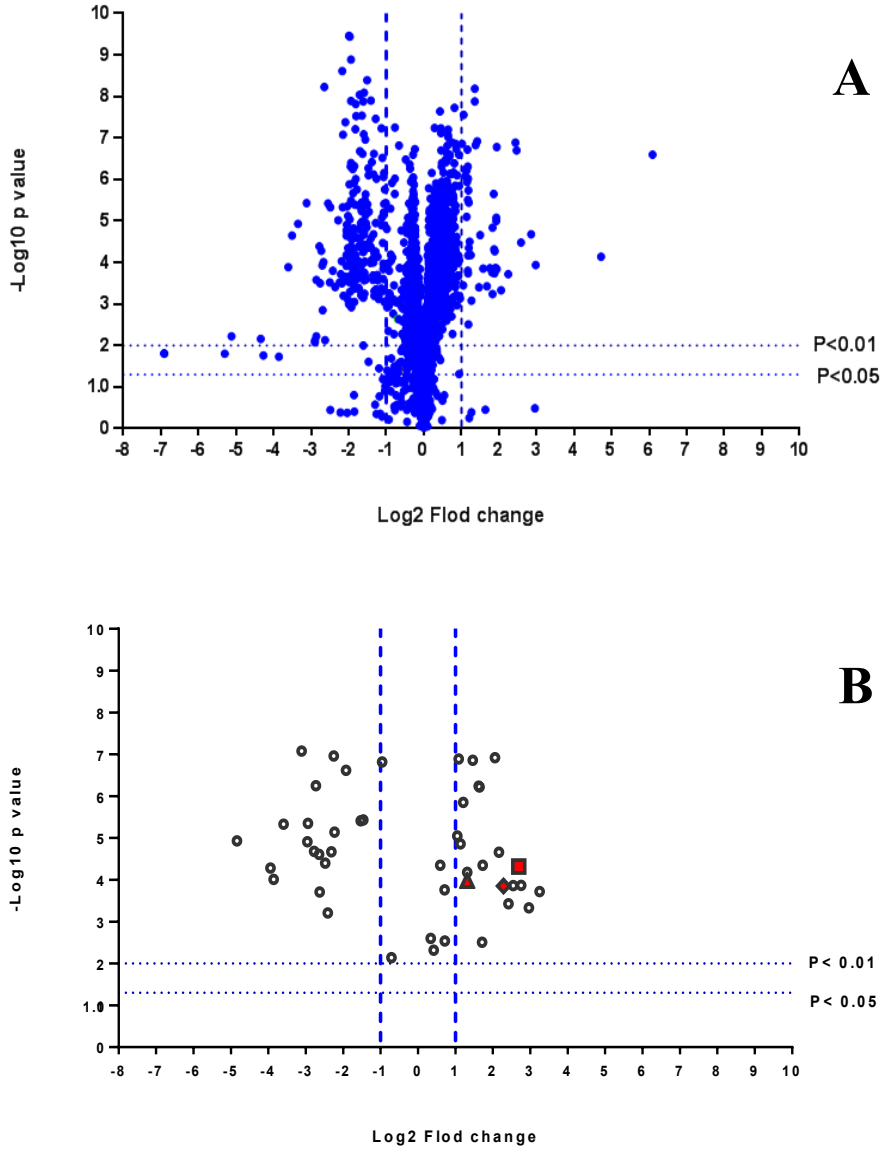

Figure 14. A volcano graph illustrating distribution of different upregulated and downregulated fragment peptides in bovine theca cells exposed to DOM-1. The log 2 fold values greater than 1 and lower than -1 represent more than 2 fold increases or decreases in phosphorylation, respectively. The Y axes indicate significance levels. Graph A illustrates all the phosphorylated proteins inside the cells. Identified proteins with significantly increased and decreased phosphorylation status are illustrated in graph B. The core MAPKs 1, 13 and 14 are shown as red triangles, diamonds and squares, respectively.

The most predominant biological functions associated with mycotoxin exposure were regulation of kinase activity and cellular response to growth factor stimulus. The most predominant molecular functions were receptor signaling protein serine/threonine kinase activity and MAP kinase activity (Table 2).

Table 2. Gene ontology annotation of major biological and molecular functions associated with proteins phosphorylated or dephosphorylated in theca cells by mycotoxin exposure.

pathway description	count in gene set	False discovery rate
Biological function:		
Regulation of kinase activity	8	0.00178
Regulation of cellular response to heat	4	0.00253
Protein folding	5	0.00269
Cellular response to growth factor stimulus	7	0.00269
Regulation of protein kinase activity	7	0.00295
Molecular function:		
Receptor signaling protein serine/threonine kinase activity	5	1.13e-05
MAP kinase activity	3	0.000311

Network analysis with STRING shows the active interactions between these signaling molecules in the form of nodes and edges (Figure 15). In this model, network nodes represent proteins and each node represents all the proteins produced by a single, protein-coding gene locus. Small nodes illustrate protein of unknown 3D structure and the large nodes illustrate proteins of known (or predicted) 3D structure. The green nodes represent the proteins whose

phosphorylation was upregulated in response to DON and DOM-1 and the red nodes represent proteins whose phosphorylation was downregulated in response to these mycotoxins. The edges represent the protein-protein associations. The blue edges represent associated from curated database and is a characteristic of gene co-occurrence. The violet edges identify genes that are homologous and are co-expressed. This STRING network shows a clear cluster of known or predicted interactions between MAPK1, MAPK13, MAPK14, EDN1, GNGT1 and YWHAB, all of which were hyperphosphorylated in response to DON and DOM-1. There was another cluster of interactions between PTGES3 and CHORDC1, EIF5A and RANBP2, involving hyperphosphorylation of CHORDC1 and EIF5A but hypophosphorylation of PTGES3 and RANBP2.

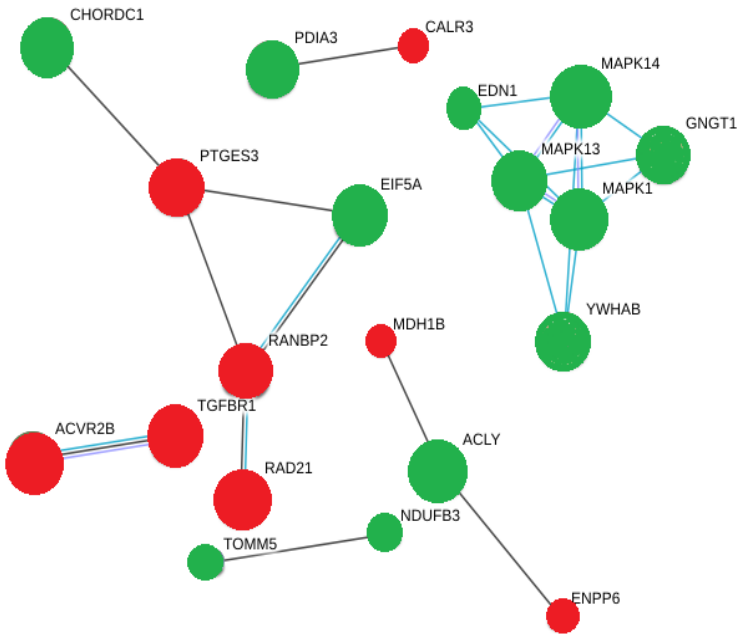

Figure 15. A string model of different intracellular signaling pathways activated by DON and DOM-1. MAPKs are the core signaling molecules in this network and have a significant effect on the cell fate due to this stimulation. Green and red nodes indicate upregulated and downregulated molecules respectively The edges are also representative of various interactions between these molecules (Szklarczyk et al., 2015).

Based on the statistical importance of these interactions, the proteins with increased phosphorylation and their functions are listed in Table 3, and the proteins with decreased phosphorylation and their functions are listed in Table 4.

Table 3. Proteins whose level of phosphorylation was increased in response to DON and DOM-1 in bovine ovarian theca cells.

Protein name	Peptide Sequence	Fold increase by DON	Fold increase by DOM-1	Function
MYCBP	TKLAQYEPPQEEKR	12	7	Stimulates activation of E-box-dependent transcription by MYC, a proto-oncogen protein
CALML4	YDEFIQKLTIPVRDY	12	9	Ca ion binding protein, correlates with MYO5A, B and 1G, involves in cell malignancy
LAMTOR4	MTSALTQGLER	5	5	An amino acid sensing molecule and activator of TORC1 family members, which are carcinogens, promotes cell growth in response to growth factors
CXCL11	TEVIITLK	4	4	A chemotactic for interlukin-activated T-cells, involves in tumor angiogenesis
MAPK1	VADPDHDHTGFLTEYV ATR	2.4	2.2	Main component of Ras/Raf/MEK/ERK cascade, mediates cell growth and survival, participates also in a signaling cascade initiated by activated KIT and KITLG/SCF.
MAPK14	HTDDEMTGYVATR	5.1	6.5	One of the four p38 MAPKs, cellular response to pro-inflammatory cytokines and physical stress
MAPK13	HTDVEMTGYVVTR	7.7	4.9	MAPK activity, one of the four p38 MAPKs, cellular response to pro-inflammatory cytokines and physical stress, activation of transcription factors such as ELK1 and ATF2
GNGT1	MPVINIEDLTEKDKLK	2.1	2.2	Signal-transducer activity, GTPase activity
EDN1	LKAQLYRDK	2.8	3.1	Positive regulation of mitotic nuclear division, protein kinase C-activating G-protein coupled receptor signaling pathway
YWHAB	VFYLKMKGDYFR	4.7	5.8	Blocks the nuclear translocation of the phosphorylated form (by AKT1) of SRPK2 and antagonizes its stimulatory effect on cyclin D1
CHORDC1	SYVTMTATKIEITMRK	3.1	2.3	Involved in stress response, regulates centrosome duplication, acts as co-chaperone for HSP90
TOMM5	EDVISSIR	2.1	1.7	Mitochondrial outer membrane translocase complex, responsible for the degradation of active cytoplasmic enzyme and organelles during nutrient starvation

EIF5A	IVEMSTSKTGK	2.2	2.3	mRNA-binding protein involves in translation elongation, regulates also TNF-alpha-mediated apoptosis
NDUFB3	DPWGRNEAWRYMGG FANNVSFVGALLK	2.5	2.9	Electron transform from NADH to the respiratory chain (ubiquitine), integral component of the membrane
ACLY	SGASLKLTLLNPKGR	2.8	2.1	Acetyl-CoA biosynthetic process, citrate metabolic process, lipid biosynthetic process
PDIA3	GFPTIYFSPANKKQNPK	2.8	3.1	Catalyzes the rearrangement of -S-S- bonds in proteins, responds to endoplasmic reticulum stress

Table 4. Proteins whose level of phosphorylation was decreased in response to DON and DOM-1 in bovine ovarian theca cells.

Protein name	Peptide Sequence	Fold decrease by DON	Fold decrease by DOM-1	Function
THEM4	SIWALRGR	-33	-33	A thioestrase that involves in mitochondrial fatty acid metabolism
PDP1	LRPQDKFLVLATDGLWETMHR	-22	-22	Catalyzes the dephosphorylation of the α-subunit of the E1 component of the pyruvate dehydrogenase complex
ST6GAL	GEDGERLYSSMSRALLR	-20	-20	Transfers sialic acid from the substrate CMP-sialic acid to galactose containing acceptor substrates from oligosaccharides
PPARG	LNHPESSQLFAKLLQKMTDLR	-10	-16	Regulates β-oxidation of fatty acids, negative regulator of cholesterol storage
HNRNPA1	VVEPKRAVSR	-7	-7	Packaging of pre-mRNA into hnRNP particles, transports poly (A) mRNA from nucleous to the cytoplasm
CALR3	GKTLIIQYTVKHEQK	-7.1	-7.7	Ca binding, cell differentiation
PTGES3	SILCCK	-5.6	-6.7	Cell proliferation, PGE synthase activity
MDH1B	ELEKESLK	-5.6	-2.7	TCA cycle, malate dehydrogense activity
RAD21	KLIVDSVKELDSK	-5.0	-6.3	Apoptotic process, cell division, RNA polymerase II transcription regulatory, region sequence-specific binding
ENPP6	HSEIYNKVRR	-5.0	-5.3	Phosphodiesterase activity, choline metabolic process, lipid catabolic process
RANBP2	SGLKDFKTFLTNDQTK	-5.0	-5.3	Regulation of gluconeogenesis, involved in cellular glucose homeostasis, ligase activity
ACVRB	SVNGGTDCLVSLVTSVTNDLPK	-4.0	-6.7	ATP binding, metal ion binding, receptor signaling protein Ser/Thr kinase activity
TGFB1	IELPTVGKPSSGLGPVLAVEEAGPVCFVCISLAMVAC	-2.4	-4.8	A receptor signaling protein with ser/thr kinase, activity, transforming growth factor beta binding, activation of MAPKK activity, pathway-restricted SMAD protein phosphorylation, positive regulator of apoptosis

Chapter 5: General discussion

This study is the first proteomic study to evaluate the impact of DON and DOM-1 on the function of theca cells in mammals. Previous studies on the function of mycotoxins employed PCR or western blot approaches to measure only preselected target genes and protein kinases, such as MAPKs that act through RSR or PKR pathways, whereas here we used a global approach to detect changes in novel and previously unsuspected signaling molecules involved in cellular response to mycotoxins. The only other study of the phosphoproteome in response to DON was performed with exposure of a macrophage cell line to toxic doses (250 ng/ml) of DON (Pan et al., 2013). The only changes in protein phosphorylation in common with the present study was for MAPK1 and MAPK14. This is not entirely surprising, as theca cells and macrophages are likely to express different genes, but illustrates that MAPK1 and MAPK14 are major pathways involved in DON action in most if not all cell types.

Analysis of biological processes and functions indicate that 30 min exposure to DON or DOM-1 activated MAPK activity and growth factor signaling pathways. This is consistent with the known ability of DON to rapidly increase phosphorylation of MAPK3/1, and demonstrated in numerous cell types including granulosa cells (Guerrero-Netro et al., 2015). However, as DOM-1 has little effect on immune and epithelial cell proliferation (S. Dänicke et al.; Sundstøl Eriksen et al., 2004), there are no published studies on any potential mechanism of action of DOM-1.

Three of the most significantly increased phosphoproteins were MAPKs: MAPK1, MAPK13 and MAPK14. MAPK1 (ERK2) is a Ser/Thr kinase which is phosphorylated by MAP2K1/MEK and MAP2K2/MEK2 on thr-185 and tyr-187 in response to external stimuli, and mediates many biological function such as cell growth, survival, differentiation and apoptosis (Johnson & Lapadat, 2002) and is a critical component of the Ras-Raf-MEK-ERK signal transduction cascade. The ERK cascade is highly upregulated in human cancers, and is typically activated by growth factor stimulation of cell surface receptor tyrosine kinases (RTKs) and other signaling molecules with known oncogenic potential. The RTKs span the

cell membrane, and the intracellular catalytic domains possess tyrosine kinase activity, catalyzing the transfer of the γ-phosphate of ATP to the OH groups of tyrosine residues

ERK1/2 are both expressed in most, if not all, mammalian tissues, with ERK2 levels generally higher than ERK1. Knock-out studies in mice showed that ERK1 has been found to specifically regulate thymocyte maturation. Downstream, activated ERK regulates growth factor-responsive targets in the cytosol and also translocates to the nucleus where it phosphorylates a number of transcription factors regulating gene expression (McCubrey et al., 2007). ERK1/2 indirectly regulates translation by inducing tRNA and rRNA synthesis. Both regulate transcription indirectly by phosphorylating the 90 kDa ribosomal protein S6 kinases (RSKs), a family of broadly expressed serine/threonine kinases activated in response to mitogenic stimuli including growth factors and tumor-promoting phorbol esters. Active RSKs seems to play an important role in transcriptional regulation by translocating to the nucleus and phosphorylating factors such as c-*fos* at Ser362, serum response factor (SRF) at Ser103, and cyclic AMP response element-binding protein (CREB) at Ser133 (Dhillon, Hagan, Rath, & Kolch, 2007).

The MAPK1 is deactivated by DUSP3 (dual-specific phosphatase 3), DUSP6 and DUSP9 which are a sub-class of the ubiquitous protein tyrosine phosphatases (PTPases) that are uniquely able to hydrolyze the phosphate ester bond on both a tyrosine residue and either a serine or threonine residue located on the same protein. Cell-division cycle 25 (CDC25) DUSPases have been shown that are attractive targets for novel anticancer agents, as they have a crucial role in controlling cell-cycle progression and genetic instability (Lyon, Ducruet, Wipf, & Lazo, 2002).

MAPK 13 and MAPK 14 are the essential component of MAPK family and are involved in cellular response to pro-inflammatory cytokines. Depending on the organ studied, they can act as pro- or anti-apoptotic factors (Gao et al., 2013; Kim et al., 2008). They are also highly expressed in many malignancies However, in response to DON and DOM exposure, these MAPKs are highly phosphorylated (6 fold) in bovine theca cells. These data are consistent with the increase in MAPK14 in granulosa cells exposed to DON, as measured by Western blotting (Guerrero-Netro et al, 2015). We are not aware of any other reports measuring the effect of mycotoxins on phosphorylation of MAPK13.

Reactome analysis suggested significant association of the MAPKs with GNGT1, EDN1 and YWHAB. Endothelin 1 (EDN1) is a G-protein and is an endothelium-derived vasoconstrictor peptide that has 2 receptors, ETR1 and ETR2. However, ETR1 activates Ras-Raf-MEK-ERK signaling pathway and the upstream signaling molecules. EDN1 receptors initiate intracellular signaling pathways leading to activation of MAPK3/1, MAPK14 and JNK1 MAPKs and consequently to nuclear targets. The activation of JNK1 seems closely related to the phosphorylation of nuclear transcription factor c-Jun (Yamboliev, Hruby, & Gerthoffer, 1998). It has been shown that EDN1 and thrombin stimulate Raf-1-independent activation of MAPK in smooth muscle cells (Shapiro, Evans, Davis, & Posada, 1996). Expression of EDN1 has not been previously reported in theca cells, therefore it is likely that the protein detected here may be derived from contaminating endothelial cells; the phosphorylation of EDN1 by DON and DOM treatment observed here may account at least in part to the increase in MAPK activity observed.

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAB), also known as 14-3-3 protein β/α, has a role in the Ras-signal transduction pathway and is a positive regulator of MAPK activity. It blocks the nuclear translocation of AKT1 and antagonizes the stimulatory effect of AKT on cyclin D1 expression, and eventually blocks apoptosis (Zheng et al., 2012). If indeed EDN1 and YWHAB are upstream of MAPK activity, these data suggest that DON and DOM-1 may act through these pathways.

Guanine nucleotide-binding protein G subunit gamma-T1 (GNGT1), or transducin γ, has GTPase activity and acts as a signal transducer in various transmembrane signaling systems. It is best known to regulate the rhodopsin-mediated signaling pathway in vision (Liu & Pan, 2016), although as a GTPase it may also regulate Ras activity. Although speculative, GNGT1 may therefore lie upstream of Ras-MAPK signaling. We can find no evidence in the literature for functional links between EDN1, YWHAB and GNGT1.

Increased phosphorylation in response to DON and DOM-1 was also observed for CHORDC1, TOMM5 and EIF5A. Cysteine and histidine-rich domain-containing protein 1 (CHORDC1), translocase of outer mitochondrion membrane 5 (TOMM5) and eukaryotic translation initiation factor 5A-1 (EIF5A) all contribute to cell proliferation and suppress signals involved in apoptotic pathways (Gano & Simon, 2010; Vogel, Read, Rehg, & Hansen,

2013; Zanelli et al., 2006), with EIF5A being involved in translation. The increase in phosphorylation of these proteins in response to the pro-apoptotic DON and DOM-1 is perplexing, and may be an initial protective response of the cells to these agents.

A number of other proteins were found to be phosphorylated in response to DON and DOM1. MYCBP (c Myc binding protein) stimulates activation of E-box dependent transcription by MYC and is a proto-oncogen protein that is involved in many malignancies (Xiong, Du, & Liang, 2010). LAMTOR4 (Regulator complex protein LAMTOR4) is an amino acid sensing molecule and activator of TORC1 family members, which are carcinogens. It promotes cell growth in response to growth factors regulates Rag GTPase function that act as a scaffold for the recruitment of mTORC1 (C.-S. Zhang et al., 2014). CALML4 is a Ca ion binding protein and correlates with uncharacterized proteins, mostly MYO5A, B and 1G proteins, and is involved in cell malignancy (Mielcarek et al., 2013). CXCL11 (C-X-C chemokine 11) is chemotactic for interlukin-activated T-cells and is involved in skin immune responses and angiogenesis in tumours (Strieter, Belperio, Phillips, & Keane, 2004). There is no clear evidence for interactions between these molecules, and the physiological role of these proteins in theca cells is obscure. We are not aware of any other reports suggesting that DON or DOM1 alter the activity of these proteins.

Some signaling molecules were dephosphorylated after addition of DON and DOM-1, including molecules involved in intracellular signaling, including TGFBR1 and ACVR2B.

Transforming growth factor-β receptor type 1 (TGFBR1) is a potent activator of MAPKK pathway and pathway-restricted SMAD protein phosphorylation. It contributes with p38 and TRAF6 autoubiquitination which ubiquitinates MAP3K7 to induce apoptosis. TGFBR1 is a potent inhibitor of epithelial and haematopoietic cell growth and proliferation (Rooke & Crosier, 2001), and inhibition of growth of lung epithelial cells by TGFB1 is directly associated with rapid stimulation of Ras-Raf-MEK-ERK signaling pathways (Jakowlew, Jacks, & Pandey, 2006). ACVR2B is a transmembrane ser/thr kinase that cooperates with SMAD2 and SMAD3 to stimulate activin-induced transcription. In the ovary, this molecule has an autocrine and paracrine role in follicular development (Shav-Tal, Lapter, Parameswaran, & Zipori, 2001). The dephosphorylation of these proteins is inconsistent with the simultaneous increase in MAPK phosphorylation observed after DON and DOM-1

treatment, although it is likely that the timing of changes of phosphorylation of these proteins is not the same as the MAPKs. The E3 SUMO-protein ligase RanBP2 (RANBP2) is an inhibitor of cell proliferation (Fahrenkrog, 2014), and has GTPase binding activity and is involved in nucleocytoplasm transport and transcriptional regulation (Geiss-Friedlander & Melchior, 2007). This also applies to double-strand-break repair protein rad 21 homolog (RAD21), which is an RNA polymerase II transcription regulator (M. Yan et al., 2012) and is involved in apoptosis via its cleavage by caspase-3 or caspase7 during early steps of apoptosis and plays a key role in chromosome cohesion during the cell cycle, in DNA repair (Pati, Zhang, & Plon, 2002).. Decreased phosphorylation of these molecules would be expected to favour proliferatory pathways, which is inconsistent with the actions of DON and DOM-1 on theca cells. The only functional link we can find between proteins hyper- and hypo-phosphorylated in response to DON and DOM-1 is between EIF5A and RANBP2; EIF5A is exported from the nucleus in association with exportin 4, and RANBP2 is part of the complex that disassembles the export complex in the cytoplasm (Lipowsky et al., 2000). It is not clear whether the phosphorylation status of these two proteins is linked.

A number of other proteins underwent significant dephosphorylation in response to mycotoxin treatment, including some involved in energy metabolism.

THEM4 (acetyl CoA thioestrase THEM4) is a molecule with thioestrase activity and involved in mitochondrial fatty acid metabolism and in apoptosis via its regulation of AKT1 signaling pathway (Brocker, Carpenter, Nebert, & Vasiliou, 2010). PDP1 (pyruvate dehydrogenase [Actyl-transferring]-phosphatase1) catalyzes the dephosphorylation and concomitant reaction of the α-subunit of the E1 component of the pyruvate dehydrogenase complex (Holness & Sugden, 2003) and is a negative regulator of the TCA cycle. ST6GAL (β-galactoside α-2,6-sialyltransferase 2) transfers sialic acid from the donor of substrate CMP-sialic acid to galactose containing acceptor substrates from oligosaccharides (Krzewinski-Recchi et al., 2003); MDH1B (putative malate dehydrogenase 1B) has malate dehydrogenase activity and plays a role in oxidoreductase activity of NAD or NADP and a main player in TCA cycle (Beeler et al., 2014); PPARG (peroxide proliferator-activated receptor-γ) regulates β-oxidation of fatty acids and is a negative regulator of cholesterol storage (Medina-Gomez, Gray, & Vidal-Puig, 2007). ENPP6 (ectonucleotide

pyrophosphatase/phosphodiestrase family member 6) is a molecule involved in choline and lipid metabolism. It is a choline-specific phosphodiesterase, that hydrolyzes glycerophosphocholine (GPC), a degradation product of phosphatidyl choline (PC), It participates in choline metabolism. ENPP6 is highly expressed in liver sinusoidal endothelial cells and has a role in developing oligodendrocytes, which actively incorporate choline and synthesize PC. It has been shown that ENPP6-deficient mice exhibited fatty liver and hypomyelination (Morita et al., 2016). The physiological impact of DON and DOM-1 on these metabolic pathways is unclear.

Other dephosphorylated proteins have roles in transcription and translation, including TFAP2A (transcription factor AP-2-α), which is a positive regulator of transcription from RNA polymerase II promoter and is a negative regulator of apoptosis and ROS metabolic pathway (Wentzel & Eriksson, 2011); HNRNPA1 (heterogenous nuclear ribonucleoprotein A1), which plays a crucial role in packaging of pre-mRNA into hnRNP particles and the transport of poly (A) mRNA from nucleous to the cytoplasm (Han, Tang, & Smith, 2010). Changes in these proteins, specifically a loss of activity, would be consistent with the known ability of toxic levels of DON to inhibit translation through the RSR.

Conclusion and future perspectives

This study has revealed that exposure of theca cells to low (sub-toxic) doses of DON and DOM-1 results in increased activation of several major MAPK signaling pathways that have not previously been reported. In addition, a number of novel protein phosphorylation events occur in response to DON and DOM-1 exposure. The levels of DOM-1 used here is within the range observed in vivo in cattle fed contaminated feed, therefore DOM-1 is likely to impact follicle function in cattle.

This study examined the effects of a single dose at a single time-point of exposure. Future experiments should be aimed at establishing a dose-response of these phosphorylation events. Perhaps more importantly, the time course of phosphorylation and dephosphorylation events should be determined. Mass spectrometry is ideal for this purpose, as western blotting is limited by the ability of available antibodies to detect the native bovine proteins, and becomes laborious when 20 or more proteins are involved. A detailed time-course may also allow the determination of the sequence of intracellular pathway activation in response to DON and DOM-1.

The bottom-up MS approach used here is however limited by the phosphoenrichment strategy. The enrichment chemistry is not infallible, and it is likely that some phospho-peptides are not efficiently retained on the titanium solid phase. An alternative approach is to separate proteins on a SDS gel and perform in-gel tryptic digestion of narrow molecular mass bands containing proteins of interest. For example, MAPK1, MAPK13 and MAPK14 are between 38 and 44 kDa in size, and could be easily isolated and examined without phospho-enrichment.

The functional biology of the putative interactions of MAPK, EDN1, GNGT1 and YWHAB warrant exploration. Initial experiments would include the use of MAPK inhibitors to determine if MAPK activation is necessary for DON-induced changes in EDN1, GNGT1 and/or YWHAB phosphorylation.

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