DANYEL BUENO DALTO

PIRIDOXINA ASSOCIADA AO SELÊNIO SOBRE A FUNÇÃO OVARIANA, DESENVOLVIMENTO EMBRIONÁRIO INICIAL E DESEMPENHO REPRODUTIVO DE FÊMEAS SUÍNAS

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Tese apresentada ao Curso de Pós -Graduação em Ciência Animal da Universidade Estadual de Londrina, como requisito à obtenção do título de doutor.

Orientador: Prof. Dr. Caio Abércio da Silva

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Londrina, 12 de dezembro de 2014

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DALTO, Danyel Bueno. **Importância da piridoxina (vitamina B6) no impacto da fonte dietética de selênio sobre alguns aspectos da função ovariana, desenvolvimento embrionário inicial e desempenho reprodutivo de fêmeas suínas**. 2014. 208 f. Tese (Doutorado em Ciência Animal - Universidade Estadual de Londrina, Londrina -Brasil).

RESUMO

Este estudo teve por objetivo deteminar os efeitos da piridoxina combinada com selênio dietético (Se) sobre o sistema antioxidante, alguns aspectos da função ovariana, desenvolvimento embrionário inicial e desempenho reprodutivo em leitoas. Três experimentos foram conduzidos usando 45, 18 e 84 leitoas, respectivamente. No primeiro experimento (45 animais), as leitoas foram distribuidas aleatoriamente em uma das cinco dietas experimentais: 1) dieta basal (CONT); 2) dieta basal + 0,3 mg/kg de Na-selenito (MSeB60); 3) dieta 2 + 10 mg/kg de HCl-piridoxina (MSeB610); 4) dieta basal + 0,3 mg/kg de levedura enriquecida com Se (OSeB60); and 5) dieta 4 + 10 mg/kg def HCl-piridoxina (OSeB610). Amostras de sangue foram coletadas no dia seguinte a cada estro e ao abate (perfil de longo prazo), e diariamente do dia -4 ao dia +3 do quarto estro (perfil peri-estral). Ao abate (dia 3 após o quarto estro), fígado, rins e ovários foram coletados e a taxa de ovulação acessada. No segundo experimento (18 animais), apenas os tratamentos CONT, MSeB610 e OSeB610 foram utilizados. Todas as leitoas foram inseminadas ao quinto estro e eutanaziadas cinco dias após para coleta de embriões. Um micro-arranjo específico para embriões suínos foi utilizado para detectar genes diferentemente expressos entre MSeB610 vs CONT, OSeB610 vs CONT e OSeB610 vs MSeB610. No terceiro experimento (84 animais), os cinco tratamentos previamente descritos foram utilizados. Amostras de sangue para os perfis de longo prazo e peri-estral foram realizadas como descrito para o primeiro experimento. Ao abate (dia 30 após o quinto estro), fígado, rins, embriões e corpos lúteos foram coletados e a taxa de ovulação acessada. De maneira geral, os resultados para o perfil de longo prazo mostram que a concentração sanguínea de Se em leitoas CONT foi menor que em leitoas suplementadas com Se ($P < 0,05$) e que OSe foi maior que MSe ($P < 0,05$). Menores concentrações de piridoxal-5-fosfato (P-5-P) nos eitrócitos foi encontrada em leitoas B60 que em B610 ($P < 0,05$). Para o perfil peri-estral (primeiro e terceiro experimento), os efeitos dos tratamentos sobre o Se sanguíneo e a P-5-P plasmática foram similares aos do perfil de longo prazo. A atividade da Se-glutationa peroxidase sanguínea (GPX) foi maior nas leitoas suplementadas com Se que nas CONT ($P < 0,05$) e MSe foi maior que OSe ($P < 0,01$), mas não para a GPX plasmática ($P > 0,27$). A concentração de Se no fígado foi menor em leitoas CONT que em suplementadas com Se ($P < 0,01$), enquanto menor concentração de Se nos embriões foi encontrada no grupo CONT que nos suplementados com Se ($P < 0,01$) e OSe foi maior que MSe ($P = 0,06$). As análises de expressão genética por PCR relevaram que *GPX1*, *GPX3*, *GPX4* e selenocisteina liase estavam fortemente expressados em leitoas OSeB610 ($P < 0,05$). As análises de micro-arranjo mostraram 10, 247 e 96 genes diferentemente expressos em MSeB610 vs CONT, OSeB610 vs CONT e OSeB610 vs MSeB610, respectivamente. A comparação OSeB610 vs CONT regulou positivamente genes relacionados com a síntese global de proteínas, mas não com a síntese de selenoproteínas. Em conclusão geral, a homeostase do Se e a atividade da GPX no sangue total em leitoas são significativamente impactados pelo nível e fonte de Se durante o período peri-estral. No entanto, a atividade da GPX no plasma sanguíneo foi afetada pela suplementação com vitamina B6. Esses resultados sugerem que a B6 dietética modula a rota metabólica do OSe para a ativação do sistema GPX e que seu efeito combinado pode estar envolvido na função

ovariana levando à otimização das condições de ovulação. Além disso, OSeB610 afeta o metabolismo do blastocisto expandido suíno mais marcadamente que MSeB610 e estimula seletivamente genes envolvidos com a defesa antioxidante. A gestação parece exercer efeitos importantes sobre as concentrações sanguíneas de Se e plasmáticas de vitamina B6 e proteínas. A transferência materna de Se para os embriões inicia precocemente na gestação. Os efeitos negativos da vitamina B6 sobre o homogeneidade da leitegada merecem estudos adicionais.

Palavras-chave: Embrião. Glutationa peroxidase. Leitoa. Ovulação. Piridoxina. Selênio.

DALTO, Danyel Bueno. **The importance of pyridoxine (vitamin B6) for the impact of the dietary source of selenium on some aspects of ovarian function, early embryo development, and reproductive performance in gilts**. 2014. 208 p. Thesis (PhD. in Animal Science - Universidade Estadual de Londrina, Londrina -Brazil).

ABSTRACT

This study aimed to determine the effects of pyridoxine combined with dietary selenium (Se) on the antioxidant system, some aspects of ovarian function, early embryo development, and reproductive performance in gilts. Three experiments were performed using 45, 18, and 84 gilts, respectively. For the first experiment (45 animals), gilts were randomly assigned to one of the 5 experimental diets: 1) basal diet (CONT); 2) basal diet + 0.3 mg/kg of Na-selenite (MSeB60); 3) diet 2 + 10 mg/kg of HCl-pyridoxine (MSeB610); 4) basal diet + 0.3 mg/kg of Se-enriched yeast (OSeB60); and 5) diet 4 + 10 mg/kg of HCl-pyridoxine (OSeB610). Blood samples were collected on the day after each estrus and at slaughter (long-term profiles), and daily from d -4 to d +3 of the fourth estrus (peri-estrus profiles). At slaughter (d 3 after the fourth estrus), liver, kidneys, and ovaries were collected, and ovulation rate assessed. For the second experiment (18 animals), only treatments CONT, MSeB610 and OSeB610 were used. All gilts were inseminated at their fifth estrus and sacrificed 5 d later for embryo harvesting. A porcine embryo-specific microarray was used to detect differentially gene expression between MSeB610 vs CONT, OSeB610 vs CONT and OSeB610 vs MSeB610. For the third experiment (84 animals), the five treatments described above were used. Blood samples for long-and peri-estrus profiles were performed as in the first experiment. At slaughter (d 30 after the fifth estrus), liver, kidney, embryos and corpora lutea were collected and ovulation rate assessed. The overall results have shown that, for long-term profile, CONT had lower blood Se than Se-supplemented gilts ($P < 0.05$) and OSe was higher than MSe ($P < 0.05$). Lower erythrocyte pyridoxal-5-phosphate (P-5-P) concentration was found in B60 than B610 gilts ($P < 0.05$). For peri-estrus profiles (first and third experiments), treatment effects on blood Se and plasma P-5-P were similar to those for long-term profiles. Blood Se-glutathione peroxidase activity (GPX) was higher in Se-supplemented than CONT gilts ($P < 0.05$) and MSe was higher than OSe ($P < 0.01$), but not for plasma GPX activity ($P > 0.27$). Liver Se concentration was lower in CONT than Se-supplemented gilts ($P < 0.01$), whereas lower embryo Se concentration was found in CONT than Se-supplemented gilts ($P < 0.01$) and OSe were higher than MSe gilts ($P = 0.06$). The PCR gene expression analysis revealed that *GPX1, GPX3, GPX4*, and selenocysteine lyase were highly expressed in OSeB610 gilts ($P < 0.05$). Microarray analysis showed 10, 247 and 96 differentially expressed genes for MSeB610 vs CONT, OSeB610 vs CONT and OSeB610 vs MSeB610, respectively. The OSeB610 vs CONT comparison up-regulated genes most related to global protein synthesis but not to selenoproteins. In general conclusion, the Se status and blood GPX activity of gilts are significantly impacted by both Se level and source during the peri-estrus period. However, the effect on plasma GPX activity was caused by B6 supplementation. These results suggest that dietary B6 modulate the metabolic pathway of OSe towards activation of the GPX system, and their combined effect may be involved in the ovarian function leading to optimal ovulation conditions. Additionally, OSeB610 affects porcine expanded blastocysts metabolism more markedly than MSeB610 and selectively stimulate genes involved with antioxidant defense. Gestation appears to have important effects on blood Se, and plasma B6 and protein concentrations. The maternal Se transfer to porcine embryos initiates early in gestation. The negative effects of B6 on embryos heterogeneity deserve further investigations.

Key words: Embryo. Gilt. Glutathione peroxidase. Ovulation. Pyridoxine. Selenium.

LISTA DE FIGURAS

Revisão de literatura

Artigos para publicação

The importance of pyridoxine (vitamin B6) for the impact of the dietary selenium sources on the antioxidant system, hormonal profile and ovulation rate in gilts

Gene expression of porcine blastocysts from gilts fed organic or inorganic selenium and pyridoxine

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The importance of pyridoxine for the impact of the dietary selenium sources on the GPX system, embryo development, and reproductive performance in gilts

LISTA DE ABREVIATURAS E SIGLAS

B6	vitamin B6
CAT	catalase
CL	corpora lutea
COQ6	Q6 monoxygenase
E2	estradiol
FAD	flavin mononucleotide
FSH	follicle stimulating hormone
GLRX3	glutaredoxin 3
GnRH	gonadrotopin realease hormone
GPX	glutathione peroxidase
GR	glutathione reductase
GRX	glutaredoxin
GSH	glutathione
GSSG	oxidized glutathione
Hb	hemoglobin
HO2*	hydroperoxyl
H2O2	hydrogen peroxide
L*	free radical
LH	luteininzing hormone
LOO*	peroxyl radicals
LOOH	hydroperoxides
Met	methionine
MSe	mineral selenium
NAD	nicotinamida-adenina-dinucleotídeo
OSe	organic selenium
O2	oxygen
O2*	superoxide radical anion
PL	pyridoxal
PLP	pyridoxal phosphate
PM	pyridoxamine
PMP	pyridoxamine phosphate
PN	pyridoxine

PNP	pyridoxine phosphate
P4	progesterone
P-5-P	pyridoxal-5-phosphate
PDXK	pyridoxine kinase
PEB	porcine expanded blastocyst
PRDX4	peroxireductase 4
ROO*	peroxyl radical
ROS	reactive oxygen species
SCLY	selenocysteine lyase
Se	selenium
Sec	selenocysteine
Se-GPX	selenium-dependent GPX
SeMet	selenomethionine
SEPSECS	Sep tRNA:Sec tRNA synthase
SOD	superoxide dismutase
TAC	total antioxidant capacity
TXN	thioredoxin
*OH	hydroxyl radical
*NO	nitric oxide

SUMÁRIO

1. INTRODUCTION

The focus on hyperprolificacy has made profound changes in swine reproduction during the last 15 to 20 years. Increasing litter sizes were achieved but at the expense of quality of ovulation, integrity of embryos and, eventually, within-litter homogeneity (Freking et al., 2007).

Post-natal strategies have been proposed to decrease within-litter weight variation but with limited success. Therefore, a pre-natal strategy appears essential but a reliable improvement of this production trait requires a better understanding of the "*in utero*" physiological causes of the phenomenon (Fortier et al., 2012). The hormonal regulation of the ovarian metabolism generates reactive oxygen species (ROS) and free radicals (Agarwal et al., 2005). Those toxic metabolites must be neutralized locally in order to maintain tissue integrity and function. The protection against ROS and free radicals occurs through antioxidants such as vitamins E and C, as well as glutathione (GSH).

Follicular cells survival is increased *in vitro* by the presence of vitamin C and selenite in the media culture (Rose et al., 1999; Murray et al., 2001), and *in vivo* effects of antioxidants on ovaries were observed in beef cattle (Sergerson et al., 1977) and sheep (Sergerson and Ganapathy, 1980), in which administrations of vitamin E maximized fertilization rates. However, in pigs, little information is available on the importance of an adequate antioxidant status during the peri-ovulation period for optimal ovulation (Fortier et al., 2012).

In recent years, the role of selenium (Se) on the body antioxidant defence system as an essential part of glutathione peroxidase (GPX) has received considerable attention

(Surai et al., 2006). Selenium is an essential trace element derived from inorganic (MSe) or organic (OSe) sources. Organic Se is deposited into proteins, following the methionine (Met) metabolism (Schrauzer, 2003), or like MSe, is incorporated into 25 selenoproteins (Windisch, 2002). Roughly half of the selenoproteins confer cellular protection against oxidative stress. Many reactions in the pathway of OSe for the control of the GPX system are pyridoxine-dependent (Yasumoto et al., 1979). Roy et al. (2011) have recently shown the importance of pyridoxine for an adequate flow of OSe towards the GPX system in response to oxidative stress pressure induced by the peri-estrus period in gilts.

In this regard, the hypothesis of this PhD research program was that the complementation of OSe with vitamin B_6 maximizes the activity of the systemic GPX system, leading to an improvement in the quantity and quality of follicles, and consequently increasing ovulation rate and embryo quality.

2. LITERATURA REVIEW

2.1 Reprodutive aspects of sows

2.1.1 Puberty

Puberty is determined by the onset of the first estrus, the so called pubertal estrus, followed by regular cycles of 18 to 24 days. For breeds commonly used in breeding programs the first estrus occurs naturally around 200 to 220 days of age, varying from 135 to 250 days of age (Hughes and Varley, 1980a).

Ramirez and McCann (1963) proposed the Gonadostatic Theory to explain puberty and this is the most accepted theory ever since. These authors showed that either mature or immature animals produce luteinizing hormone (LH) in their pituitary gland. Moreover, the synthesis and secretion of this hormone is enhanced after gonadectomy in rats. When these rats were treated with estradiol (E_2), plasma and pituitary concentration of LH were reduced, but this response was amplified in immature rats. The hypothalamus-pituitary axis of immature animals is more sensible to small doses of E_2 and this hormone inhibits the secretion of both gonadotropin releasing hormone (GnRH) and LH. During the period just before puberty the hypothalamus sensibility to the negative effects of E_2 is reduced (Christenson et al., 1985) and thereafter, GnRH and LH secretion are stimulated, with further ovulation (Evans and O'Doherty, 2001). Thus, the hypothalamus-pituitary axis sensibility to the inhibitory effects of E_2 would be the main factor influencing puberty.

2.1.2 Estrus cycle

The estrus cycle is characterized by shifts in the concentration of reproductive hormones, mainly LH, follicle stimulating hormone (FSH), E_2 and progesterone (P_4), triggering anatomic and behavioral changes.

Senger (2003) divided the estrus cycle into follicular and luteal phases by considering the major structure taking place in the ovary. The follicular phase begins with the regression of the corpora lutea (CL) and remains until ovulation. During this phase there is predominance of E_2 with growing follicles in the ovaries. The luteal phase lasts from ovulation until the regression of the CL, a period in which the CL are predominant in the

ovaries and P_4 is the main hormone. The estrus cycle can either be divided in four steps that are the pro-estrus, estrus, metestrus and diestrus.

The pro-estrus is characterized by growing follicles and a higher estrogens production by these follicles. The high levels of estrogens lead to anatomical and behaviour changes (e.g. vulvar edema and secretion, hyperemia, and interest on the boar), lasting 1 to 3 days (Anderson and Einarsson, 1980). Estrus is the period when the dam allows to be mated by the boar. Almond and Dial (1990) suggested that high levels of E_2 block the release of LH by a negative feedback but, upon reaching a certain level, a positive feedback of E_2 triggers the estrus symptoms and thereafter the pre-ovulatory LH surge (Sesti and Britt, 1993). The LH surge will set off ovulation and then luteinisation of granulosa cells and inner teca (Ainsworth et al., 1990). The ovulation usually occurs, in average, 35 hours after the onset of estrus (Soede et al., 1995).

The metestrus begins just after the estrus and lasts 2 to 3 days. During this period, E_2 concentrations are very low and the follicles begin to be transformed into CL that will produce P_4, preparing the uterus for gestation (Kolb, 1979). Finally, the diestro lasts 7 to 12 days and is characterized by the maximum production of P_4 by the CL between the days 12 and 14 of the cycle. The luteal regression takes place between the day 15 and 16 in non-pregnant sows and P_4 concentration is basal at 17 to 18 days, characterizing the beginning of a new cycle. In pregnant sows, the CL keep producing P_4 which avoids the development of new follicles (Clark et al., 1982).

2.1.3 Follicular dynamics

During ovarian follicle development different patterns of cell proliferation are observed and can be classified in two stages: gonadotropin independent and gonadotropin

dependent. During gonadotropin independent phase, the primordial follicles grow but become atretic because, according to Foxcroft et al. (1994), the absence of gonadotropin stimuli stops follicles development. The process of recruitment and selection of follicles is gonadotropin dependent and once selected, the pool of follicles will grow to ovulatory size and the others will become atretic.

Pulses of LH with high frequency and low amplitude associated to a low level of P_4, appears to be necessary for the recruitment to begin, but FSH is responsible for the growth of the follicles. About 50 follicles make up the recruited group, but the small and medium follicles (< 6.5 mm) will undergo atresia. During the recruitment phase, development of aromatase activity in the granulosa cells is a key event, leading the recruited follicles to produce E_2 (Driancourt, 2001).

Follicles selection begins at about day 14 to 16 of the cycle, with a remarkable change in the follicle dependency of FSH for LH. During this phase, the follicles that will ovulate grow in diameter from 4-5 mm to 9-11 mm (Gordon, 1997b), and the follicular LH is critical for final maturation and ovulation rate (Knox, 2005). Afterwards, LH is involved in inducing ovulation and in formation of the CL.

The ovulated follicles undergo morphological changes to form the hemorrhagic corpus and further the CL, a process called luteinisation (Senger, 2003). After ovulation there is a reduction in diameter to 4-6 mm but when the CL formation is completed, it grows to 8-9 mm on day 7 of the cycle (Hughes and Valley, 1980b). Simultaneously, important functional changes occur, as the shift from E_2 to P_4 production (Foxcroft et al., 1994), along with the progressive functionality of the CL. The higher levels of P_4

stimulate the uterus to prepare and maintain an appropriate environment for embryo development.

2.1.4 Conceptus development

Passing through the *cumulus oophorus*, performing the acrosome reaction, and penetrating the zona pellucida, the spermatozoid will be in the peri-vitelline space near to the oocyte membrane. The fusion between them occurs between the oocyte plasma membrane and the plasmatic membrane covering the equatorial part of the sperm head. Oocyte activation, due to sperm fusion, comprises morphological and biochemical changes as sodium input, hyperpolarization of the membrane, enhancement of intracellular calcium, and exocytose of cortical granules (Prather, 2001). Thereafter, the formation and migration of the two pronucleus, DNA synthesis, and changes in protein synthesis occurs (Yanagimachi, 1994).

Once formed, both pro-nucleus migrate to the center of the oocyte. Their nuclear membrane and nucleolus disappear, a process called syngamy (Senger, 2003), and both maternal and paternal chromosomes gather for the diploid number of the specie, completing the fecundation (Crozet, 1993).

Just after fertilization the mitotic cells division begins, with increasing number of cells but embryo size. During the first 6 to 8 hours after ovulation the embryo, at the two-cells stage (Gordon, 1997a), is found in the oviduct. Around 48 hours after ovulation embryos are at the four-cells stage and begin to migrate towards the uterus, a process that lasts up to 24 hours. When a compact mass is formed, and it is no longer possible to determine the number of cells, the embryo is called morula. As the embryo grows these

cells begin to separate into two groups, the internal and the external cells which will generate the trophoblast and inner cellular mass. Both group of cells form different junction between their surrounding cells and once completed, a diffusion of liquids trough the zona pellucida occurs, leading to the formation of a cavity filled with liquid, the blastocoel (Senger, 2003). At this stage, the embryo is called blastocyst. As the embryo grows the blastocoel continues to expand pressing the trophoblastic cells against the zona pellucida, and at this stage the embryo is called expanded blastocyst. Thereafter, the zona pellucida is disrupted and the hatched blastocyst grows faster, migrates through the uterus and becomes dependent of uterine conditions to survive (Martinat-Bottea et al., 1998). Development is not a uniform process among embryos so, embryos can be found in different stages and different sizes in polytocous species like swine. The overall process from ovulation until the blastocyst stage is presented in Figure 1.

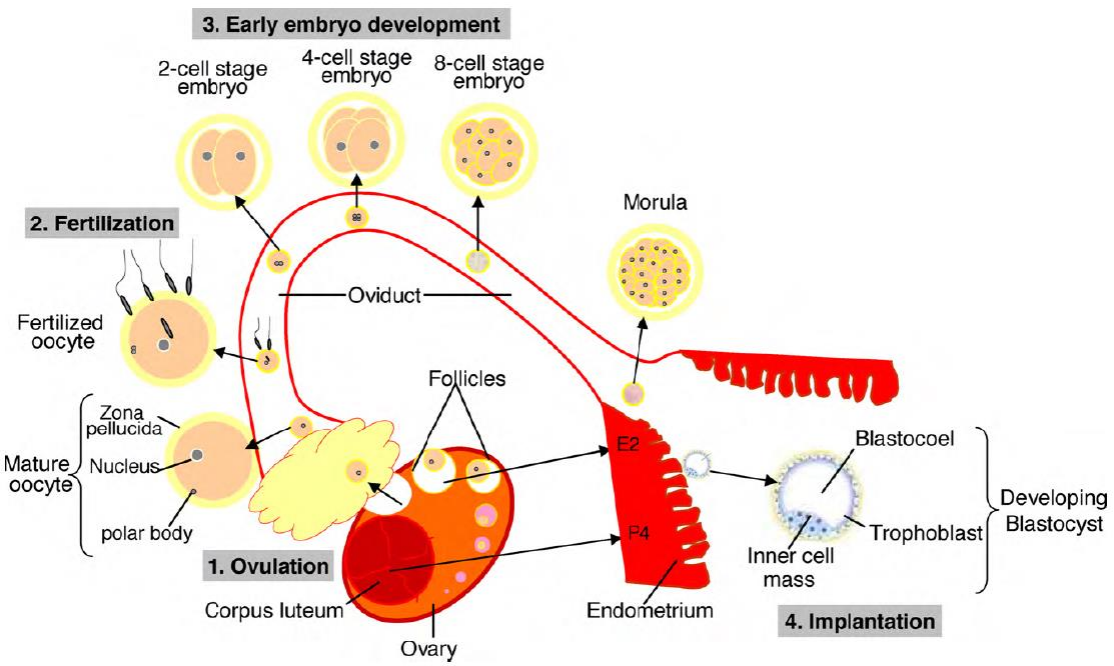


Figure 1 - Diagram of the mammalian female reproductive organs, early embryonic development and implantation (Adapted from Al-Gubory et al., 2010).

Embryo migration is a continuous process occurring until day 12 of gestation. On day 13, the embryo begins to establish a contact with the endometrium (Dantzer and Winther, 2001) and this contact becomes closer until day 25-26, when the process of embryo-endometrium interaction and placentation is concluded. During this first stage of gestation, nutritional requirements are not high and neither the sow nor the embryos build body reserves.

The fetal period in swine begins around 35 days of gestation when all the organs are completely formed and calcium begins to be deposited in the bones. Many factors impair fetal development as genetics, maternal nutrition, litter size, uterine capacity, placental efficiency for nutrients transport, and hormonal factors (Ashworth et al., 2001). During this period, sows and fetuses will build body reserves and, between days 90-100, a phase of substantial increase in nutrient exchange between sow and fetuses takes place (Biesen et al., 1998). McPhersom et al. (2004) showed that fetus weight increase exponentially during gestation, but after day 80 of gestation this increase is accentuated and nutritional requirements are then higher than at any other period of gestation.

2.2 Selenium and pyridoxine

2.2.1 Selenium

2.2.1.1 Overview

Selenium was discovered in 1817 by Berzelius and its necessity for biochemical and physiological processes was established by Schwarz and Foltz in 1957. In the last decades, research has identified Se as an essential nutrient for animal nutrition, health and

178 well-being (Surai, 2006), and the requirements for animals and humans have been
179 reported (FDA, 2004).

180 Although this microelement acts as part of many hormones and enzymes influencing
181 the activity of all organs and tissues (Beckett, 1993; Karunasinghe et al., 2004), the major
182 role of Se in the body is related to the antioxidant system (Surai et al., 2006). Selenium
183 acts in cell functions including growth, control of apoptosis, maintenance of cellular
184 redox state, and DNA-reparation (Rooke et al., 2004).

185 All these functions lead to an important role of Se in animal reproduction. In many
186 species, high deposition of Se was found in endocrine glands (Vohra et al., 1973) and
187 reproductive organs (Allan et al., 1999), and a role in growth and development of porcine
188 embryos in early gestation was demonstrated (Fortier et al., 2012).

189

190 **2.2.1.2 Sources, requirements, and bioavailability**

191 There are huge variations in Se status of human and animal populations around the
192 world due to wide variations on soil Se levels and, consequently, food and feed Se levels
193 (Reilly, 2006). Plants absorb Se from soil as selenite or selenate and synthesises
194 selenoamino acids, with selenomethionine (SeMet) as the major selenocompound
195 (Whanger, 2002). Indeed, yeast cells and some bacteria can uptake selenite or selenate
196 from the media and synthesize selenoamino acids (Combs, 2001).

197 Food is the major source of Se with seafood, cereals, and meat products containing
198 the higher concentrations, whereas low levels are found in milk, vegetables, and fruit
199 (Tinggi et al., 1992; Tinggi, 1999). Dietary Se comes from both organic (mainly SeMet)
200 or inorganic sources (selenite or selenate) (Dumont et al., 2006), and both can be added

into commercial diets for domesticated species. Selenite is naturally present in phytoplankton and, historically, it has been the main source of Se added to nutritional supplements (Whanger, 2002). In the wild however, animals normally ingest Se from plants in the form of SeMet (Combs and Combs, 1986). According to Kim and Mahan (2001), both dietary MSe and OSe are toxic at doses higher than 5 mg of Se per kg of diet in pigs, but selenosis effects are more severe and occur sooner with the mineral source.

In order to ensure an adequate and secure Se intake, many countries have established dietary guidelines (BNF, 2001; FDA, 2004; Levander and Burk, 2006). According to NRC (2012), the dietary Se requirement ranges from 0.3 mg/kg for weanling to 0.15 mg/kg for finishing pigs and sows. However, it has been reported that Se requirement increases during pregnancy and lactation due to a higher demand for maternal transfer to conceptuses and newborns (Smith and Picciano, 1986). Therefore, currently there are growing interests in study the effects of supra-nutritional Se supplementation in animals and humans.

The bioavailability of Se is dependent upon the chemical forms in which it is absorbed and metabolized, but in general, OSe is more bioavailable than MSe (Rider et al., 2010). Since the first approval of selenium-enriched yeast (organic source) to be used in animal diets (FDA, 2001), comparisons between both OSe and MSe have been made.

Selenomethionine is the major form of OSe (Whanger, 2002) and is considered the most appropriate source for human and animal nutrition (Schrauzer, 2003), with high rates of absorption, tissue accumulation, antioxidant bioavailability, and lower toxicities and environmental pollution (Swanson, 1991; Vendeland et al., 1994). However, its metabolic fate can be modulated by the pyridoxine status (Pavlata et al., 2011). In fact, a

low intake of pyridoxine could impair OSe utilization and, consequently, increase Se requirements (Yin, 1992). Sodium selenite, the most common source of MSe, has some shortcomings such as potential toxicity at high dietary level (Kim and Mahan, 2001a), low absorption efficiency (Mahan and Parret, 1996), and potential oxidative effects (Spallholz, 1994).

The main sources of Se to meet the high requirements of young rapidly growing pigs are placental transfer and colostrum/milk ingestion (Mahan and Moxon, 1977). Therefore, Se supplementation during gestation should meet not only the maternal requirements but its transfer to the littler. Tissue Se concentration of newborns are positively correlated with both maternal Se concentration and Se source fed to the sows (Mahan and Peters, 2004), in which OSe is more efficiently deposited in tissues (Mahan, 1994) and transferred to colostrum and milk (Mahan and Kim, 1996; Surai, 2002).

Although Se concentrations in developing fetal tissue have been reported for cattle (Abdelrahman and Kincaid, 1995) and sheep (Langlands et al., 1982), little data are available for porcine fetus.

2.2.1.3 Metabolism

In pigs, Se is not absorbed in the stomach. At intestinal level, Se sources differ in the pathway and rate of absorption, but both MSe and OSe sources are preferentially absorbed in the ileum, followed by jejunum, cecum, and colon (McDowell, 2003).

Selenite is passively absorbed by simple diffusion (Wolfram, 1989), whereas OSe is actively absorbed through amino acid transport mechanisms (McDowell, 2003). The post-absorbed MSe is quickly transformed into selenide (hydrogen selenide; H_2Se) and

247 then converted by selenophosphates into functional selenoproteins containing
248 selenocysteine (Sec) (Windisch, 2002). Regarding OSe, it is either directly metabolized
249 to reactive forms of Se or follows the Met metabolism (Sunde, 1984). Because the Met-
250 tRNA cannot discriminate between Met and SeMet, using both interchangeably in protein
251 synthesis, OSe is effective in building Se reserves in tissues (Schrauzer, 2003).

252 One of the most important functions of Se is the biosynthesis of Se-dependent GPXs
253 (Se-GPX). Both Se sources activate these enzymes, but by different pathways.
254 Additionally, many reactions in the pathway of OSe towards Se-GPX are pyridoxine-
255 dependent (Yasumoto et al., 1979).

256 Inorganic Se, obtained from the diet as selenite, undergoes a thiol dependent
257 reduction to selenide, with further catalysis of selenophosphate syntethase to
258 selenophosphate. The last is the Se donor to the exchange of the phosphate moiety of Ser-
259 tRNA for Se, generating a Sec-specific tRNA[Ser]Sec. This special tRNA, that contains
260 the Sec insertion sequence (SECIS), along with a Sec-tRNA-specific elongation factor
261 (eEFSEC), and a specific SECIS binding protein (SECISBP2), is essential for Sec
262 incorporation into selenoproteins, as Se-GPX (Johansson et al., 2005).

263 Absorbed OSe (SeMet), in turn, receives an adenosine group from ATP, a reaction
264 catalyzed by S-adenosyl-Met synthetase, to produce S-adenosyl-Met. This enzyme
265 transfers the methyl group to an acceptor molecule with further hydrolysis to Se-
266 homocysteine. This compound has two primary fates: re-methylation to SeMet by
267 tetrahydrofolate or transsulfuration to Sec. The first and second steps of the
268 transsulfuration pathway are catalyzed by cystathionine β-synthase and cystathionine γ-
269 lyase, respectively (Mosharov et al., 2000), both pyridoxine-dependent enzymes.

Selenocysteine may also contribute to the synthesis of GSH, but this is negligible compared to the global GSH pool synthesized from sulfur-cysteine (Osawa et al., 1992). It can be also reduced to selenide, via selenocysteine lyase, which will follow the same steps as MSe (Figure 2).

It is noteworthy to mention that for OSe metabolism, high levels ROS not only promote a negative feed-back on the re-methylation of SeMet from Se-homocysteine, but also promote a positive feed-back on the transsulfuration pathway. This control does not occurs in the selenite pathway (Mosharov et al., 2000).

It is commonly assumed that a Se storage and transport system should exist to handle its distribution in tissues by adjusting plasmatic Se levels (Yin, 1992). Selenium concentrations vary widely in different tissues, with liver as the major body storage site in pigs (Seboussi et al., 2010). Much of Se in liver is associated with selenoproteins (Driscoll and Copeland, 2003). According to Ghany-Hefnawy et al. (2007), Se quantification in maternal liver is considered the best method for determining the Se status in both the dams and fetuses. Maternal hepatic Se levels in sows are greater than in the offspring (Mahan and Kim, 1996), with a negative correlation between maternal and fetal liver Se levels, and a positive correlation between maternal liver and plasma Se concentrations, indicating a withdrawal of maternal Se to be transferred to the fetus (Ghany-Hefnawy et al., 2007).

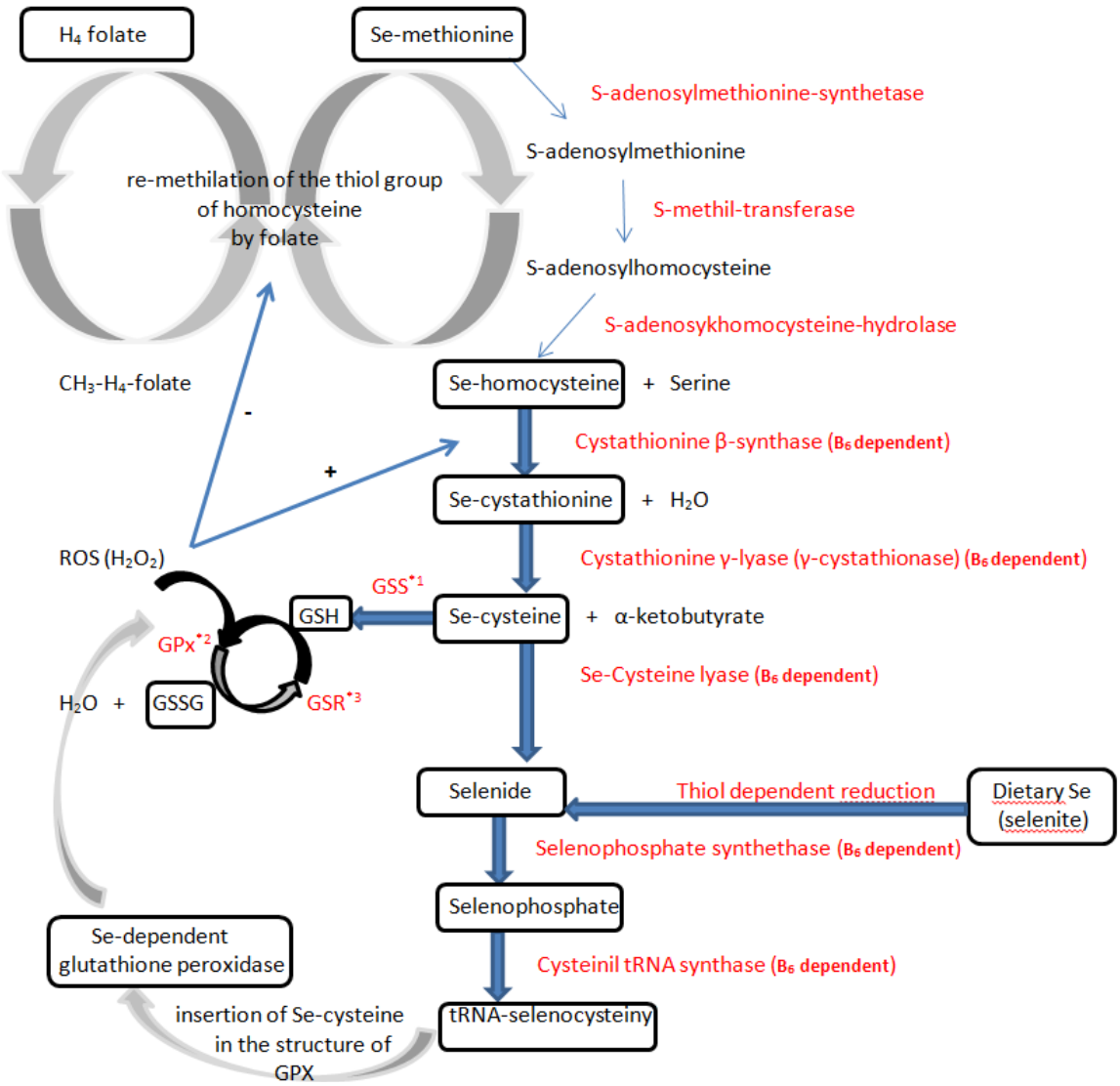


Figure 2 - Selenomethionine transsulfuration pathway and synthesis of glutathione peroxidase (Adapted from Fortier et al., 2012).

Both plasma and erythrocytes Se concentrations increase as dietary Se levels increase, with erythrocytes containing the higher concentrations (Kim and Mahan, 2001b). According to Giguère et al. (2005), whole blood Se possibly better reflected the Se status of the animal because more than 50% of the overall circulating Se is present in erythrocytes, mainly bound to hemoglobin.

Maternal Se intake during gestation affects neonatal tissues Se concentration in a dose-dependent manner (Kim and Mahan, 2001b), with the highest levels in kidney, intermediate in liver and pancreas, and lowest in muscle, thymus, and thyroid gland (Zhan et al., 2011). Selenate transport across placental membrane vesicles has been described as an anion exchange pathway shared with sulphate (Shennan, 1988). In contrast, selenoamino acids are actively transported from dam to fetus (Jacobssons and Oksanenh, 1966).

An increased concentration of Se in the allantoic fluid is found within gestation, indicating not only an increased transfer of Se from the dam to the fetus but also a greater excretion by the fetus (Hamdy et al., 1963). The absence of correlation between amniotic fluid and maternal plasma Se concentrations suggests that the fetus meets its own Se requirements regardless plasmatic maternal levels (Ghany-Hefnawy et al., 2007).

Selenium transport into colostrum and milk occurs primarily through secretory vesicles associated with casein in the mammary alveolar epithelial cell (Allen and Miller, 1981). The relative response in terms of Se concentration in milk is much greater than in blood, probably because milk protein contains more Met than blood (NRC, 2001). Additionally, colostrum Se concentrations have been shown to be greater than those of milk (Stowe et al., 1988), possibly as a result of the higher protein content.

316 According to Mahan and Peters (2004) the incorporation of MSe into colostrum and
317 milk is lower than of OSe. Supplementing sows with a combination of both Se sources
318 (0.15 mg/kg of each) resulted in Se concentrations similar to the exclusive 0.15 mg/kg
319 OSe supplementation. The higher levels of Se in colostrum, milk, and serum of dams fed
320 OSe resulted in better Se status of the litter at birth (Yoon and McMillan, 2006) and at
321 weaning (Mahan and Kim, 1996).

322 Selenium is excreted from the body via urine, feces, colostrum and milk, and
323 potentially, exhaled air (Boldizarova et al., 2003; Spears, 2003; Pavlata et al., 2005).
324 Although biliary secretion of Se can account for 28% of Se intake (Langlands et al.,
325 1986), most of it is reabsorbed and the remainder contributes to fecal endogenous losses.

326 The main homeostatic regulation of Se metabolism in the organism occurs through
327 renal excretion. Renal Se excretion is known to be influenced by level and source of
328 dietary Se, along with the physiological status of the animal (Thomson, 1998),
329 glomerular filtration of Se-compounds, and the concentrating / diluting function of the
330 kidneys (Oster and Prellwitz, 1990; Leng et al., 2000). Total Se recovery from urine and
331 feces for both selenite and selenate (82-95%) in humans indicates that retention of MSe is
332 very low, whereas ingested OSe recovery was only 26% (Thomson, 1998).

333 Excess MSe absorbed from the digestive tract and not utilised for selenoprotein
334 biosynthesis is methylated and excreted (Itoh ans Suzuki, 1997). Selenide is metabolized
335 to methylselenol and dimethylselenide, which are secreted via urine as
336 trimethylselenonium ion and selenosugar compounds (Suzuki et al., 2005). Studies with
337 Se perfusion in sheep (Árová et al., 2003) showed a decline of 50% in plasma and whole
338 blood Se levels within 4 h post-infusion, suggesting a rapid elimination from the body.

For dietary OSe, as for all amino acids, it is almost completely reabsorbed from kidney glomerular filtrate already in the first tubular segment of the nephron-proximal tubule. Reabsorbed OSe is captured by kidney capillaries and re-enters the whole body metabolism via the blood stream (Robinson et al., 1997) (Figure 3).

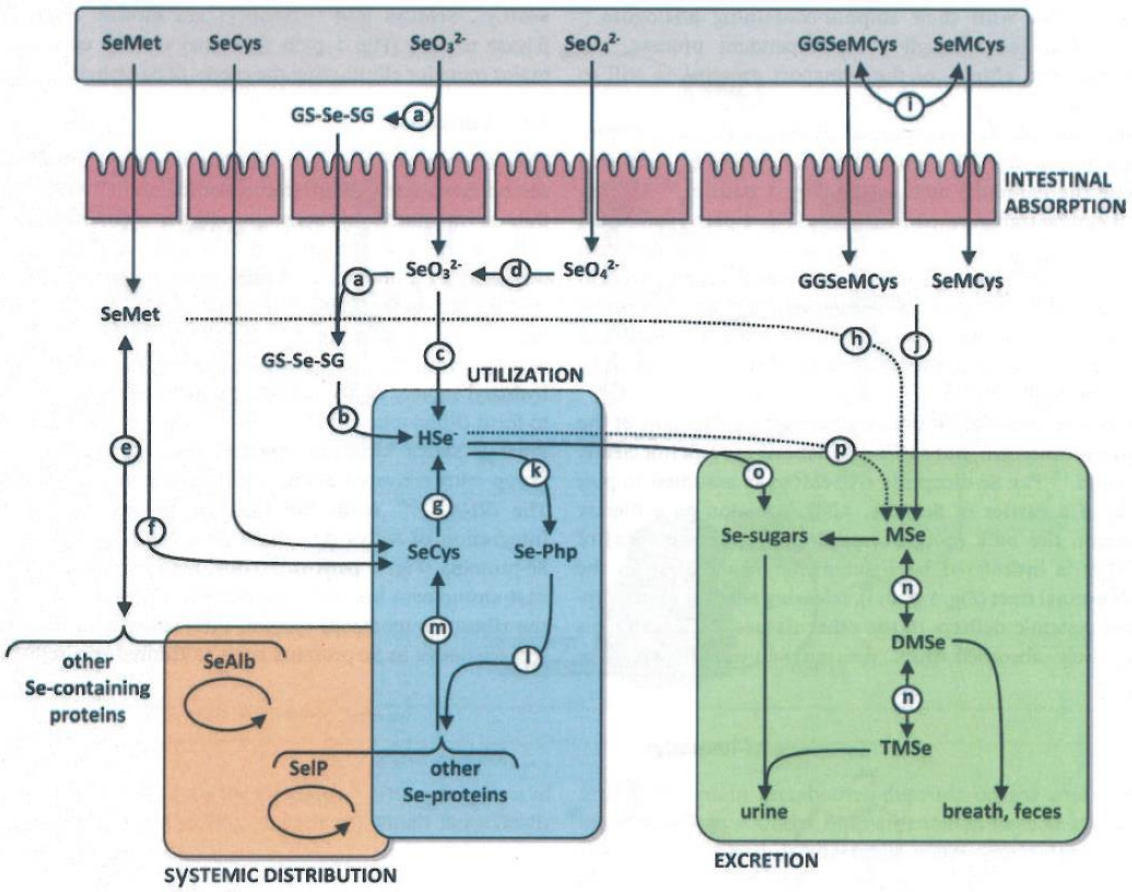


Figure 3 - Global view of selenium metabolism in mammals (Adapted from Roman et al., 2014).

2.2.1.4 Main functions

In mammals, the biological activities of Se include antioxidant defense (Steinbrenner and Sies, 2009), activation and degradation of thyroid hormones (Köhrle and Gärtner,

2009), immunity enhancement (Carlson et al., 2010), and involvement in cell growth and development (Papp et al., 2007). Therefore, proper Se intake would have positive effects on animal growth performance by improving health, but excessive Se intake leads to negative effects (Kim and Mahan, 2001b).

The current literature considers that Se status does not exert pronounced effects on thyroid hormones or thyroid-stimulating hormone levels in populations with adequate Se intake or moderate deficiency (Köhrle et al., 2005). However, the relationships between Se status and gonadal function (Köhrle et al., 2005), and between gonadal and thyroid function (Krassas, 2000) reveals the action of Se on thyroid functions.

Some biochemical studies suggest that sex hormones may influence blood Se levels during the reproductive cycle (Ohwada et al., 1996). In humans, Ha and Smith (2003) observed that blood Se parameters fluctuated during the menstrual cycle and were greatest in the peri-ovulatory phase, and Smith et al. (2000) found a positive correlation between plasma Se and E_2. In pigs, Fortier et al. (2012) showed that peri-estrus plasma FSH levels tended to change with different dietary sources of Se.

Furthermore, some observations in human and animals showed interrelations between sex hormones and the selenoprotein GPX. In rats, apoptosis in pre-ovulatory follicles induced by oxidative stress was prevented by FSH through the stimulation of follicular GPX synthesis (Tsai-Turton and Luderer, 2006); whereas in pigs supplemented with OSe, Fortier et al. (2012) showed that the different responses of E_2 around estrus were related to the GPX response. Glutathione peroxidase was also positive correlated with P_4, acting in the degradation of peroxides produced in the CL and supporting P_4 production (Kamada and Hodate, 1998).

374 Additionally, the hormonal regulation of the ovarian metabolism generates ROS and
375 free radicals (Agarwal et al., 2005), which must be neutralized locally to maintain tissue
376 integrity and function. Therefore, nutritional strategies to increase Se concentrations in
377 the body could potentially improve fertility, as shown by Rose et al. (1999) and Murray
378 et al. (2001), in which the survival of follicular cells was increased *in vitro* by adding
379 vitamin C and Se in the media culture.

380 According to Fortier et al. (2012), it appears that Se supply is transiently insufficient
381 for GPX homeostasis shortly after ovulation in Se-unsupplemented gilts, whereas it was
382 maintained and enhanced throughout the peri-estrus period by dietary supplementation
383 with MSe and OSe, respectively. These same authors also found that the GPX activity in
384 the CL was greater in Se-supplemented than in Se-unsupplemented gilts, and the CL
385 protein content were greater in OSe than MSe gilts.

386 An important application of Se relates to its potential to protect the developing
387 embryo from peroxidation during embryogenesis (Surai, 2000). The mammalian fetus
388 relies completely upon the dam to supply adequate nutrients for growth and development
389 (Hidiroglou and Knipfel, 1981) and therefore, maternal nutrition directly affects fetal
390 development (Heap, 1967). Selenium is required by the fetus to ensure numerous
391 developmental activities, including cell division and differentiation, DNA replication,
392 protein synthesis, steroid hormone binding to DNA, and oxidative defense (Bedwal and
393 Bahuguna, 1994). Fortier et al. (2012) found that the average length and weight, and
394 DNA and protein content of porcine embryos were greater in OSe than MSe treated gilts.

395 Other studies in pigs indicated that although sows serum GPX activity was not
396 affected by dietary Se source, differences in the transference of Se to their progeny were

observed (Yoon and McMillan, 2006; Svoboda et al., 2008; Fortier et al., 2012). According to Zhan et al. (2011), when compared with sodium selenite, SeMet has a higher potential of transferring antioxidants from the dam to its offspring, consequently, maternal Se supplementations is essential for antioxidant protection of the progeny (Pappas et al., 2008).

Several reports indicated that maternal OSe intake have little effect on improving litter growth performance when compared to MSe (Mahan and Peters, 2004; Yoon and McMillan, 2006; Svoboda et al., 2008), however, Hu et al. (2011) suggested that the elevated antioxidative status of dams, which is extended to its offspring, responded for the improved pre-weaning growth performance observed in piglets born from OSe supplemented sows. In growing-finishing pigs, a number of reports (Mahan and Parrett, 1996; Mahan et al., 1999; Wolter et al., 1999; Tian et al., 2006) have failed to show the effects of Se supplementation on growth performance, regardless dietary Se sources or levels. However, Zhan et al. (2011) reported that OSe was superior to improve the antioxidant status of finishing pigs when compared with MSe.

2.2.2 Pyridoxine (vitamin B_6)

2.2.2.1 Overview

The high productivity of modern swine production leads to an intense anabolism to maintain growth, gestation, and lactation at optimal rates and, therefore, vitamin requirements have to be adjusted to these production levels. Surprisingly, much of the limited information available on this topic was published more than 30 years ago.

The six vitamin B_6 (B_6) metabolically active forms (pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and their phosphorylated forms PN phosphate (PNP), PL phosphate (PLP) and PM phosphate (PMP)) are considered to have approximately equivalent biopotency (Ball, 2006a), differing between food sources, chemical structure, and metabolism. The oxidized form, 4-pyridoxic acid, is also present in the metabolism although metabolically inactive (Matte, 1997).

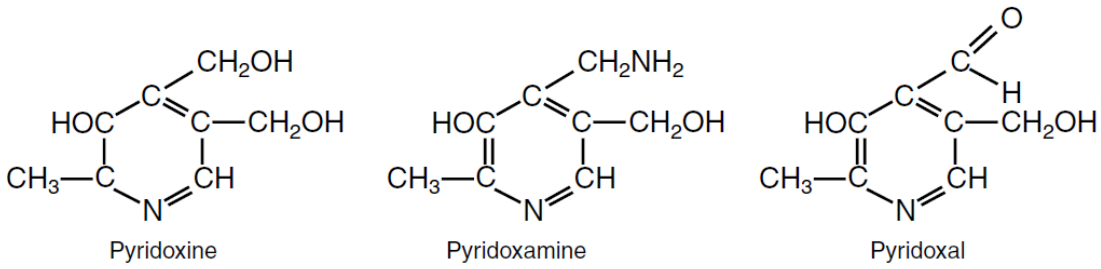
Pyridoxine is a water soluble vitamin necessary for normal growth, development, and biological functions. Due to its stability, PN hydrochloride (3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine) is used in multivitamin supplements and food fortification (Dove and Cook, 2001). Pyridoxal-5-phosphate (P-5-P), the physiologically active form of B_6, acts mainly as a cofactor for many essential enzymes, catalyzing more than 140 reactions. Additionally, it belongs to five (oxido-reductases, transferases, hydrolases, lyases, isomerases) of the six enzyme classes defined by the Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, which implies its important metabolic functions (Mooney et al., 2009).

Besides the function as a cofactor, B_6 is thought to act directly as a protective agent against ROS, such as singlet oxygen (Chen and Xiong, 2005). This underlines the wide variety of chemical reactions that B_6 promotes in the organisms and shows the importance of this vitamin.

2.2.2.2 Sources, requirements, and bioavailability

Fungi, plants, archaea, and most eubacteria are able to synthesize B_6 but most animals lack this ability and rely upon external supply. The dietary forms of this vitamin (PN, PL, and PM) are biologically inactive and naturally occurring (Figure 4).

444



445

446 Figure 4 - Chemical structure of the three naturally occurring dietary forms of pyridoxine
447 (Adapted from Mooney et al., 2009).

448

449 Vitamin B_6 is found in natural unprocessed foods with yeast extract, wheat bran, and
450 liver containing the highest concentrations, however, whole-grain cereals, nuts, lean
451 meat, fish, kidney, and vegetables are important sources (FSA, 2002). The chemical
452 forms of B_6 vary among foods of plant and animal origin. Roth-Maier et al. (2002)
453 reported that B_6 in all tested foods of plant origin occurred in as PN and PM, except for
454 maize where over than 50% of the B_6 content occurred as PL. In raw animal tissue, the
455 major form is PLP (Ball, 2006b).

456 As swine cannot synthesize B_6 as most B-complex vitamins, it must be obtained from
457 exogenous sources, through intestinal absorption. The intestine is exposed to B_6 from diet
458 and via bacterial B_6 synthesis in the large intestine (Allgood and Cidlowski, 1991).
459 However, the B_6 synthesized by the colonic microflora is not locally absorbed, thereby
460 non-coprophagous animals obtain no benefits from this source of B_6 (Combs Jr., 2012).

461 The bioavailability of B_6 ranges between 40 to 80% in different foods. Yen et al.
462 (1976) determined that 38 to 45% and 58 to 62% of PN in maize and soybean meal are
463 bioavailable, respectively. A wide variety of dietary fibers such as polysaccharides,
464 lignin, cellulose and pectin did not adversely affect B_6 availability (Nguyen et al., 1981a,

b; Nguyen et al., 1983). However, protein level, source, and quality have been shown to affect B_6 requirements. Daghir and Shah (1973), feeding 15, 20 or 25% protein diets for chicks, found a higher requirement of B_6 to increase body weight. Furthermore, an increased serine (Aboaysha and Kratzer, 1980) or Met supplementation (Kazemi and Daghir, 1971) also increases B_6 requirements for growth.

The current estimated PN requirement (NRC, 2012) for 3 to 5, 5 to 20, and 20 to 120 kg pigs are 2.0, 1.5, and 1.0 mg of B_6/kg of diet, respectively. Although no specific requirement is available for pigs during gestation or lactation, Russell et al. (1985) suggested that the daily requirement of B_6 for sexually mature gilts may be higher than 2.1 mg/day. However, it is noteworthy that, because of the limited number and outdating of these studies (ARC, 1981; INRA, 1984; NRC, 1998), their suitability for modern genetic lines and production systems might be questionable.

The B_6 content of grain-soybean meal diets (4.0 to 6.4 mg/kg) is generally adequate to meet the requirements for swine (NRC, 1998). Wilson et al. (1993) showed no effects of supplemental B_6 in nursery, grower or finisher diets based on corn and soybean meal. Woodworth et al. (1998) supplemented 2.2 to 3.3 mg of PN/kg of diet for weanling pigs and observed improved performance from 0 to 14 days post-weaning. These requirements are possibly linked to the fact that the cellular levels of the 10 nonessential amino acids are achieved and maintained through reactions catalyzed by P-5-P. Indeed, in B_6 deficiency, protein formation does not proceed normally due to poor nonessential amino acids synthesis (Guyton, 1991).

As B_6 is fairly widespread in foods, problems of primary deficiency are not prevalent. Regarding toxicity, it appears to be relatively low, although at high doses (150 mg / kg of BW) it has been shown to induce sensory neuropathy in dogs (Hoover et al., 1981).

2.2.2.3 Metabolism and functions

The small intestine (particularly the jejunum), is the main absorption site of B_6, which is a dynamic process involving several inter-related events (Wozenski et al., 1980).

The dephosphorylated forms (PN, PL and PM) are rapidly absorbed by passive diffusion and, as intestinal mucosal cells have PN kinase and PNP oxidase, there is a net accumulation by metabolic trapping (Gregory, 1997). Within the enterocyte, PN, PL, and PM are converted into their corresponding phosphates by the catalytic action of cytoplasmic PL kinase. The phosphorylated vitamers formed in the cell are largely dephosphorylated by nonspecific phosphatases, thus permitting easy diffusion of B_6 compounds across the basolateral membrane (Middleton, 1985). Much of the ingested PN is released into the portal circulation as PL (after dephosphorylation at the serosa surface) and further taken up by the liver.

Liver is the central organ for B_6 metabolism, containing all of the enzymes involved on its inter-conversions. The major forms of B_6 in that organ are PLP and PMP, which are maintained at fairly constant intracellular concentrations in an endogenous pool that is not labile. In the same organ, a second and readily mobilized pool provides B_6 for metabolic conversion and releasing into the blood (Shane, 1978). In the liver, the inactive forms are phosphorylated by PL kinase, yielding P-5-P, PNP and PMP. The latter two

508 forms are then oxidized by a flavin mononucleotide (FAD)-dependent oxidase to yield P-
509 5-P (Wada and Snell, 1961).

510 Pyridoxal-5-P does not cross cell membranes, therefore the uptake and efflux in
511 tissues most occurs in the form of PL. Much of the free P-5-P in the liver is hydrolyzed to
512 PL, which is released and circulates bound both to albumin (Anderson et al., 1974) or
513 hemoglobin in erythrocytes (Mehansho and Henderson, 1980). Once PL crosses the cell
514 membrane, biologically active P-5-P is regenerated through the action of PL kinase. Free
515 PL either leaves the cell or goes through oxidation, primarily in the liver, by the FAD-
516 dependent aldehyde oxidase and/or nicotinamida-adenina-dinucleotídeo (NAD)-
517 dependent aldehyde dehydrogenase, to yield 4-pyridoxic acid (Gregory, 1997). Small
518 amounts of PL are also excreted in urine, although much of the active B_6 is resorbed in
519 the kidney tubules (Bender, 2003) (Figure 5).

520 The total body pool of B_6 is of the order of 60 mmol (250 mg); 15 mmol (3.7 mg) per
521 kg BW, with muscle containing most (70-80%) of the body's B_6 in the form of PLP
522 bound to glycogen phosphorylase. Protein binding is thought to protect PLP from
523 hydrolysis while providing storage of the vitamin (Gregory, 1997). Muscles B_6 reserves
524 do not appear to function as a true reserve because it is unavailable to other tissues.
525 Muscle PLP is released into the circulation (as PL) only under starvation, when muscle
526 glycogen reserves are exhausted (Bender, 2003). The second largest PLP reservoir is the
527 liver, accounting for 5-10% of all body's B_6. Within this organ, most of the PLP is found
528 in the cytosolic compartment, where the metabolic conversion of dietary precursors to
529 PLP takes place (Hamm et al., 1980).

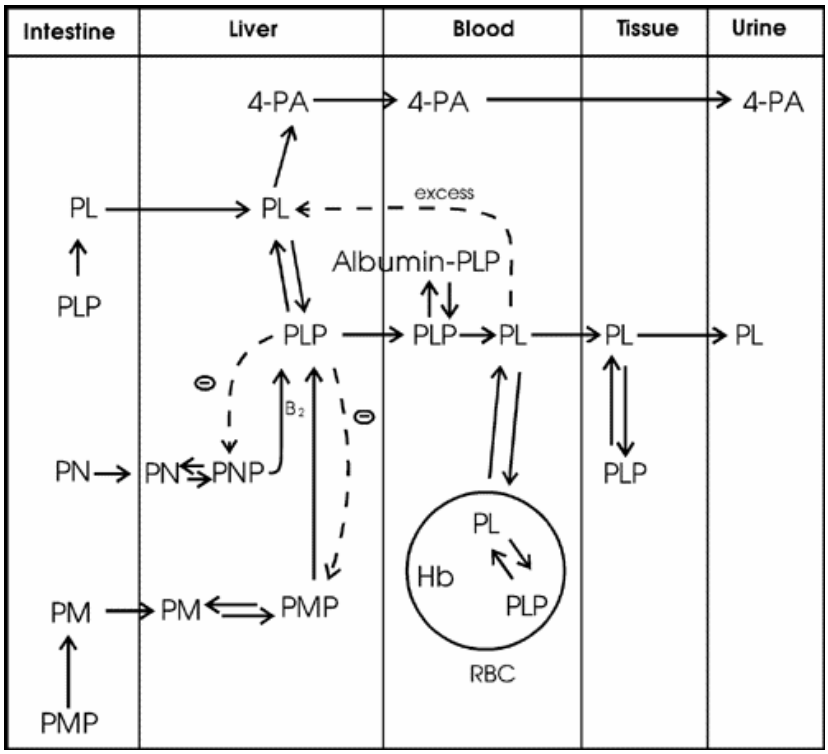


Figure 5 - Vitamin B_6 metabolism and transport (Adapted from MindExperts, 2013).

Little information is available on colostrum and milk B_6 content in sows. Bowland (1966) presented values of 2.5 µg/100mL for colostrum and 20.0µg/100mL for milk. Using a rat modelling, Benedikt et al. (1996) showed that milk B_6 consisted of 79.5% PL, 15.6% PM, and 4.9% PN at day 7, and of 79.8% PL, 13.9% PM and 6.3% PN at day 13 of lactation.

Pyridoxal-5-phosphate is historically known for its central role in amino acid metabolism, acting as coenzyme for a variety of reactions, including transamination, transsulfuration, and decarboxylation (Bender, 2003). However, it also represents an important cofactor in degrading stored carbohydrates, in which P-5-P-dependent glycogen phosphorylase mediates the glycogen breakdown and release of glucose from glycogen (Helmreich, 1992). In fatty acid metabolism, P-5-P enzyme δ-6-desaturase

544 catalyzes the synthesis of polyunsaturated fatty acids by the desaturation of linoleic and
545 linolenic acid (Nakamura and Nara, 2004).

546 This vitamin is also involved with nervous system functions (Allgood and Cidlowski,
547 1991), hemoglobin synthesis and function (Combs Jr., 2012), and a role in steroid
548 hormones metabolism was suggested (Allgood and Cidlowski, 1991). In addition, B_6 may
549 play a crucial role in protecting cells against oxidative stress, exhibiting an antioxidant
550 activity that even exceeds that of vitamins C and E (Ehrenshaft et al., 1998; Bilski et al.,
551 2002).

552

553 **2.3 Oxidation**

554

555 **2.3.1 Mitochondrial electron transport chain**

556 Oxidants are compounds capable of oxidising target molecules by abstraction of a
557 hydrogen atom or an electron, or by adding oxygen (O_2) (Lykkesfeldt and Svendsen,
558 2007).

559 Oxidation is the transfer of electrons from one molecule to another, in which the
560 electron-donating molecule is the reducing agent and the electron-accepting molecule is
561 the oxidizing agent (Agarwal et al., 2012). In the mitochondrial membrane, electrons are
562 transferred from a substrate via a series of enzymatic catalyzed steps, having O_2 as the
563 final electron acceptor.

564 The electron transport chain is the cellular mechanism used for extracting energy
565 from sunlight during photosynthesis in plants and from redox reactions such as oxidation
566 of sugars (respiration) in animals. The electron transport chain transfer electrons from an

electron donor (e.g. NADH) to an electron acceptor (e.g. O_2) by transferring H^+ ions (protons) across a membrane, resulting in chemical energy in the form of ATP. In mitochondria, the conversion of O_2 to hydrogen peroxide (H_2O_2), NADH to NAD^+, and succinate to fumarate are required to generate the proton gradient (Karp, 2008).

Four major groups of oxidation-reduction enzymes participate in these reactions, which are (1) the pyridine-linked dehydrogenases, (2) the flavin-linked dehydrogenases, (3) iron-sulfur proteins, and (4) cytochromes. The entire process is called oxidative phosphorylation, since H^+ oxidation provides energy for the phosphorylation of ADP to ATP (Eberhardt, 2001).

Oxidative phosphorylation occurs in and on the inner membrane of the mitochondria and provides ATP not only to this compartment but also to the rest of the cell. In essence, the process links the energy released by the respiratory chain to that needed to synthesize ATP. However, not all of this energy is trapped and part is released as heat. The efficiency in which the energy is captured as chemical energy or released as heat energy is determined by a number of factors (composition of the diet, nutritional status, endocrine status, genetics, etc.) (Berdanier, 2005).

The components of oxidative phosphorylation are divided into five complexes. Complexes I, III, and IV pump protons into the space between the two mitochondrial membranes. In Complex I (NADH dehydrogenase) two electrons are removed from NADH and transferred to ubiquinone. The reduced product, ubiquinol, diffuses within the membrane and four H^+ are translocated across the membrane, producing a proton gradient (Garret and Grisham, 2010). In Complex II (succinate dehydrogenase) additional electrons are delivered into the quinone pool originated from succinate and transferred

via FAD, to ubiquinone (Cooper, 2000). In Complex III (cytochrome bc_1 complex), the quinone-cycle contributes to the proton gradient by an asymmetric absorption/release of protons. Two electrons are removed from ubiquinol and sequentially transferred to two molecules of cytochrome c. The two other electrons sequentially pass across the protein where the quinone part of ubiquinone is reduced to quinol. A proton gradient is formed by two quinol oxidations (Garret and Grisham, 2010). In Complex IV (cytochrome c oxidase) four electrons are removed from four molecules of cytochrome c and transferred to molecular O_2, producing two molecules of water (Figure 6). Simultaneously, four protons are removed, contributing to the proton gradient (Cooper, 2000))

A small percentage of electrons do not complete the whole process, undergoing premature electron leakage and, consequently, generating ROS.

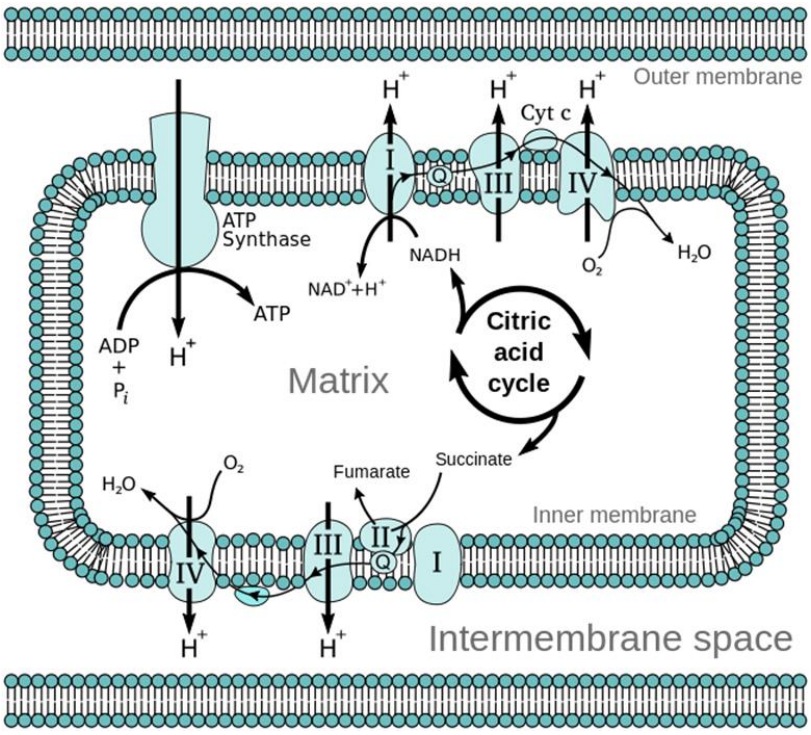


Figure 6 - Mitochondria respiration chain (Wikipedia, public domain).

2.3.2 Free radicals and reactive oxygen species

Free radicals are highly reactive agents containing at least one unpaired electron. This condition makes them greedy for electrons, to obtain an electronic stability. In biological systems, oxidation (electron loss) or reduction (electron acceptation) constitutes the two main routes for the development of free radicals (Pourova et al., 2010). Among this group of compounds the metabolites of O_2 can be classified as radicals, with superoxide radical anion (O_2*-), hydroperoxyl (HO_2*), hydroxyl radical (*OH), peroxyl radicals (ROO*), and nitric oxide (*NO) as the most important (Eberhardt, 2001).

Reactive oxygen species can be defined as O_2-containing molecules that are more reactive than the O_2 molecule present in air (Noguchi and Niki, 1999). Reactive oxygen species include free radicals as well as reactive compounds without unpaired electrons in their outer orbit. They occur naturally within cells, during any aerobic process, and are formed by electron leakage from membranes and inadequately coupled reactions; thereby the released electrons reduce molecular O_2 to O_2*-.

Superoxide radical anion is the main free radical produced during normal respiration in mitochondria and by autoxidation reactions.

$$O_2 \xrightarrow{\text{metabolism}} O_2{}^{*-}$$

These metabolites do not cause immediate damage to lipid membrane bilayers (Kruidenier and Verspaget, 2002). However, it is a precursor of other more dangerous ROS.

The hydroperoxyl radical is in equilibrium with O_2*- in aqueous solution and is formed through the transfer of a proton to an O_2 atom.

aqueous solution

$$O_2{*}^- + H_2O \longleftrightarrow HO_2{*} + OH$$

Hydroperoxyl radicals can act as oxidants in many biologically reactions and may be an important initiator of lipid peroxidation (Surai et al., 2006).

The hydroxyl radical is formed through the Fenton reaction (dismutation of peroxide catalysed by Fe^{2+}). Superoxide can participate in this reaction by donating an electron.

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

Fenton reaction

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + {*}OH$$

This is the most reactive and dangerous ROS, damaging any biological molecule. However, its action is restricted to the site of formation due to its very small half-life (10^{-9} second) (Yu, 1994).

The most important effect of free radicals on cellular metabolism derives from its impact in lipid peroxidation. In these reactions, a free radical (L*) formed from a precursor molecule (e.g. PUFA) reacts with O_2 to form peroxyl radicals (LOO*).

$$L{*} + O_2 \longrightarrow LOO{*}$$

The resulted LOO* react with other lipids producing lipid hydroperoxides (LOOH) and L*.

$$LOO^* \longrightarrow LOOH + L^*$$

Additionally, metallic ions can react with LOOH producing LOO*.

$$LOOH + Fe^{3+} \longrightarrow LOO^* + Fe^{2+} + H^+$$

2.3.3 ROS in physiological condition

Initially, ROS were considered to be potentially harmful by-products of cell metabolism. However, during the last 20 years, they have been recognised as important messengers in numerous intra-cellular pathways, maintaining and modulating a wide spectrum of vital physiological functions.

Reactive oxygen species are continuously produced within the body and, at low levels, play important physiological roles as second messengers capable of regulate apoptosis, activate transcription factors, and modulate the expression of genes involved in immune response (Hensley et al., 2000; Valko et al., 2007).

These reactive compounds and their by-products are created through various physiological and biochemical processes, with the mitochondria, endoplasmic reticulum, nuclear membrane, and cellular plasma membrane, the major intracellular sites of ROS production (Agarwal et al., 2005). The activation of phagocytes, prostaglandin biosynthesis (Parke and Parke, 1995), and enzymatic pathways such as NADPH oxidase,

xanthine oxidase, cyclooxygenases, lipoxygenases, monooxygenase, and cytochrome P450 systems, are potential extra mitochondrial ROS sources (Al-Gubory et al., 2010).

In normal physiology, ROS have important functions in cell respiration, phagocyte defense system, thyroid gland, signalling pathways, fertilization, apoptosis, ontogenesis, ageing process, male and female reproduction systems, and others (Pourova et al., 2010). Conversely, at high concentrations ROS induce oxidative stress, which have been reported to cause cell membrane damage, DNA fragmentation in somatic cells, and cell apoptosis (Tatemoto et al., 2000; Bedaiwy et al., 2004).

2.3.4 Oxidative stress and its damages

The term ‘‘oxidative stress’’ was first mentioned by Helmut Sies (1986), referring to the imbalance that arises when oxidants changes the normal redox status of major tissue antioxidants, especially GSH. Ever since, this term has been vaguely defined.

In essence, it is described as a disturbance of the equilibrium between antioxidants and oxidants in favour of oxidants (Sies, 1991). Oxidative stress might occur when an increased oxidant load overcomes the antioxidant defence system, or due to a reduced antioxidant supply (Cutler, 2005). Virtually all cellular components may undergo oxidation in presence of high concentrations of ROS, but the intra-cellular origin and the reactivity of the oxidant molecule, as well as the location and the biochemical properties of the target molecule, play a determining role on their interactions (Buettner, 1993).

The toxic effects of ROS are due to their ability to initiate cell membrane lipid peroxidation resulting in damage to cell membranes and structures (Machlin and Bendich, 1987). Reactive oxygen species not only induce direct damage to biomolecules,

699 such as DNA, proteins, membrane lipids, and carbohydrates but also indirectly alter
700 and/or deregulate cellular signaling events and consequently interfere in gene expression
701 (Halliwell and Whiteman, 2004).

702 Molecular mechanisms of ROS toxicity and ROS-mediated disease include the
703 oxidation of vital thiol compounds to disulphides, loss of tissue GSH, impairment of
704 energy generation, inhibition of Ca_2+ transport and electrolyte homeostasis, oxidation of
705 cytochromes, DNA strand cleavage, and the initiation and promotion of mutations and
706 carcinogenesis (Parke, 1994). Such cellular perturbations appear to be markedly
707 important in inflammatory conditions, in which increased oxidant production can further
708 enhance the inflammatory process (Valko et al., 2007).

709 The exposure to oxidants from a variety of sources has led organisms to develop a
710 series of defence mechanisms including preventative and repair mechanisms, and
711 physical and antioxidant defences (Cheeseman and Slater, 1993). The support against the
712 oxidative burden can be achieved by enhancing the endogenous antioxidant levels, for
713 example, by increasing dietary antioxidant supply (Young and Woodside, 2001).

714

715 **2.4 Antioxidants**

716

717 Antioxidants display many different properties in biological systems. Consequently, it
718 is difficult to find a precise description for the term ‘‘antioxidant’’. Overall, it refers to a
719 broad range of substances with the ability to neutralize free radicals by donating one
720 electron and/or H^+, preventing or delaying oxidation of a substrate (Packer, 2005).

721 The presence of powerful antioxidant defences in the organism points to the potential
722 importance of protecting biomolecules against oxidative damage. The antioxidant system

consisting of vitamins, carotenoids, flavonoids, trace elements, and enzymes and proteins, such as superoxide dismutases (SOD), catalases, GSH, and thioredoxin peroxidases (Pincemail et al., 2007). Additionally, indirect antioxidant functions are mediated by enzymes that restore endogenous antioxidant levels; such as GSH reductase (GR) that replenishes the GSH levels by reducing the oxidized GSH (GSSG) (Van den Berg et al., 2005).

Antioxidants can act in different ways, preventing free radical formation (e.g., by metal chelation), scavenging free radicals, stopping propagation of free radicals (acting as a chain-breaking), as part of the redox antioxidant network, and/or regulating gene expression (Packer, 2005). However, these reactions are protective only if the by-products formed are less reactive or damaging than the initial species.

The main redox regulation system in the body is the GSH system, employing NADPH as an ultimate electron donor and containing an NADPH-dependent enzyme and a small redox-reactive peptide. The oxidoreductase agent is GR that acts reducing the small tripeptide GSH, which in turn controls the cellular redox status. Other components of this system are glutaredoxin (GRX), a small protein that can specifically remove GSH from mixed disulfides formed between GSH and other molecules, and GPX, which reduces peroxides in a GSH-dependent manner (Su and Gladyshev, 2005).

2.4.1 Dietary antioxidants

The animal diet provides a range of different compounds that possess antioxidant activities. Since these dietary antioxidants are exogenous in nature, their intake levels can be manipulated by dietary supplements and diet modifications (Basu, 1999).

746 The most prominent representatives of dietary antioxidants are ascorbate (vitamin C),
747 tocopherols (vitamin E), carotenoids, and flavonoids. Other important biofactors to the
748 antioxidant defense include lipoic acid, coenzyme Q10, and the various metals
749 (micronutrients) such as Se, copper, zinc, manganese, and iron, which are essential for
750 the activity of many antioxidant enzymes (Packer, 2005).
751 These compounds can act as antioxidant itself or through an intricate network of non-
752 enzymatic and enzymatic antioxidants.
753

754 **2.4.2 Non-enzymatic antioxidants**

755 The non-enzymatic antioxidative defence mechanisms consist of substances with
756 different chemical characteristics, but with neutralizing ROS activity in common.
757 Examples of these are vitamin E, vitamin C, GSH, urates, ubiquinones, dipeptide like
758 carnosine, and some trace elements such as Se.
759 Glutathione (or γ-l-glutamyl-l-cysteinylglycine) is the most abundant non-protein-
760 bound thiol-containing compound found in cells (Smith et al., 1996; Valko et al., 2006).
761 It is a major component in cellular antioxidant systems, detoxifying endogenous radical
762 species. Indeed, studies indicate that protection against radical species, specifically O_2
763 radicals, is a primary function (Cooper et al., 2005).
764 This tripeptide is synthesized in the cytosol in two steps that require ATP. Firstly, γ-
765 glutamylcysteine is formed from glutamate and cysteine by the activity of γ-
766 glutamylcysteine synthetase. Further, GSH is formed by the activity of GSH synthetase,
767 which uses γ-glutamylcysteine and glycine as substrates. The formation of γ-
768 glutamylcysteine is the rate-limiting reaction in GSH synthesis, however, this reaction is

769 feedback inhibited by GSH itself, a mechanism responsible for the regulation of cellular
770 GSH concentration (Marí et al., 2009). Virtually all mammalian cells have the capacity to
771 synthesize GSH.

772 The antioxidant function of GSH is mediated by the redox-active thiol group that
773 becomes oxidized when GSH reduces target molecules. The importance of GSH as a
774 cellular redox buffer is underscored by the fact that it displays a low redox potential and
775 is found in high concentration in the cells (Han et al., 2006; Koehler et al., 2006; Marí et
776 al., 2010). Currently, it is widely accepted that GSH acts not only as a reducing agent and
777 a major antioxidant within the cells, but also as a mediator of many other physiological
778 reactions including the metabolism of xenobiotics, thiol disulfide exchange reactions, and
779 cellular signaling (cell-cycle regulation, proliferation, and apoptosis).

780 Despite its exclusive synthesis in the cytosol, GSH is present in intracellular
781 organelles including the endoplasmic reticulum, nucleus, and mitochondria. Glutathione
782 is observed predominantly on its reduced form, except in the endoplasmic reticulum
783 where it is found mainly as GSSG, which provides the adequate environment necessary
784 for disulfide bond formation and folding of nascent proteins (Hwang et al., 1992). In
785 nucleus, GSH is necessary for DNA repair and expression (Valko et al., 2007) and
786 contribute with DNA synthesis (Holmgren, 1977). Additionally, the redox balance of
787 GSH (GSH/GSSG) in cells has become a biological indicator of oxidative stress and
788 disease progression (Go and Jones, 2005; Jones, 2006).

789 Glutathione is one of the most important water-soluble antioxidants, not only because
790 of its action as a scavenger, but mainly as an indispensable factor for proper catalytic
791 action of some antioxidative enzymes.

2.4.3 Enzymatic antioxidants

The most important antioxidant enzymes include catalase, Se-GPX, and SOD (Fridovich, 1995). Because the levels of these antioxidants are determined by their rate of synthesis, they are not easily manipulated.

Trace-elements such as Se, copper and zinc play important catalytic roles for the enzymatic activity of GPX, in which one mole of protein contains one mole of Se (Ursini et al., 1985), and SOD with copper and zinc-dependent SOD (Maughan, 1999; Mates, 2000). The catalytic activity of these enzymes allows the transformation of O_2*- into H_2O_2 and H_2O, thereby inactivating important quantities of oxidants.

The cascade of endogenous antioxidant enzymes requires vitamins and energy to maintain the living system in the reduced state. For example, GR maintains tissue GSH in the reduced state at the expense of reduced NADP and FAD. Glutathione peroxidases, which the synthesis is controlled by pyridoxine-dependent reactions (Yasumoto et al., 1979), reduce soluble and membrane-bound peroxides to the corresponding alcohols at the expense of GSH which is oxidized to GSSG (Stoytcheva and Berry, 2009) (Figure 7).

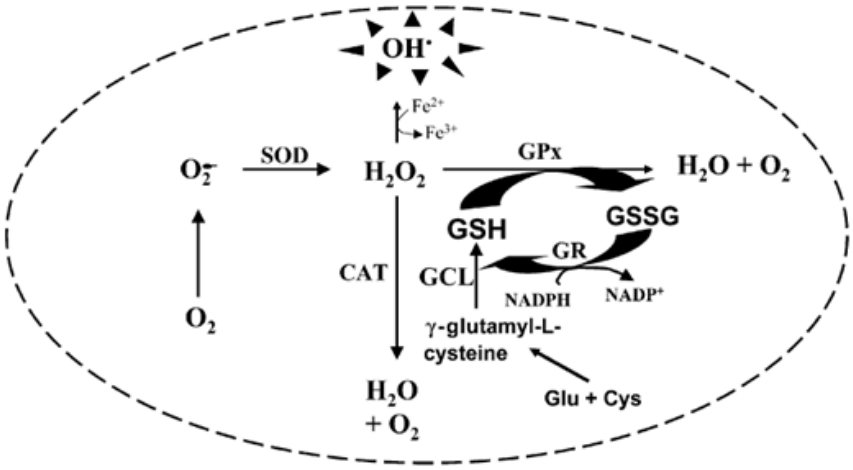


Figure 7 - Cellular enzymes responsible for eliminating ROS or potentiating the antioxidant capacity (Adapted from Surh, 2005).

Superoxide dismutases constitute the first enzymatic step that plays a vital role in the control of cellular O_2*- production. Copper-, manganese-, and zinc-containing SOD (SOD1, SOD2, and SOD3) catalyse the dismutation of O_2*- into H_2O_2 and O_2 (McCord et al., 1971) as follows:

$$2\ O_2{}^{*-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

The control of H_2O_2 production is the second enzymatic step for the control of ROS propagation. Catalase which is found within peroxisomes (Chance et al., 1979), catalyses the conversion of H_2O_2 to H_2O as follows:

$$2\ H_2O_2 \xrightarrow{\text{CAT}} 2\ H_2O + O_2$$

Because mitochondria lack CAT, the metabolism of H_2O_2 is accomplished mainly by GPX, not only in mitochondria but also in the cytoplasm (Mills, 1957). Glutathione peroxidase catalyzes the reduction of H_2O_2 and organic LOOH as follows, where L can be hydrogen, a free fatty acyl group, cholesterol, a steroid, an ester of one of these groups, or free bases of DNA or RNA:

$$2\ GSH + H_2O_2 \xrightarrow{\text{GPX}} GSSG + 2\ H_2O$$

$$2\ GSH + LOOH \xrightarrow{\text{GPX}} GSSG + H_2O + LOH$$

Eight isoforms of GPX are presently known. Among them cytosolic (GPX1), gastrointestinal (GPX2), plasmatic (GPX3), and phospholipidic (GPX4) isoforms contain a Sec in their structure (Behne and Kyriakopoulos, 2001), whereas GPX6 contains a cysteine in rodent, but a Sec in human (Kryukov et al., 2003). Non-Sec containing isoforms GPX5, GPX7, and GPX8 are also present in mammalian tissues (Nguyen et al., 2011). Both types of GPX enzymes, Se-dependent and Se-independent, have been shown to catalyze the above reactions and, therefore, protect against radical damage by reducing peroxides.

Glutathione peroxidase 1 is the major isoform and is localized mainly in the cytosol, with small fraction also present in the mitochondrial matrix, acting as the primary defense against oxidative damage to mitochondrial membranes (Marí et al., 2010). Glutathione peroxidase 1 mRNA is expressed throughout all the stages of mouse embryogenesis (Baek et al., 2005); however, GPX1-knockout mice show no abnormality in phenotype (Ho et al., 1997).

Glutathione peroxidase 2 is located mainly in crypts bases, which coincides with the location of proliferating stem cells. Interestingly, growth and differentiation of intestinal stem cells is regulated by the Wnt pathway (Pinto et al., 2003) and GPX2 expression via Wnt signals (Kipp et al., 2007; Kipp et al., 2012), suggesting a role of GPX2 in the continuous self-renewal of intestinal epithelium and mucosa homeostasis.

Glutathione peroxidase 3 is considered a secretory glycoprotein representing the overall GPX activity in plasma. In humans and mice, GPX3 has been found in blood plasma, breast milk, and fluid lavage from lung to amniotic fluids (Avissar et al., 1991, 1994, 1996). Most of its activity is observed in the proximal tubules of kidney, whereas

862 the sites of GPX3 mRNA synthesis are lung, heart, and intestine (Avissar et al., 1996;
863 Kingsley et al., 1998). The high amount of GPX3 in plasma might point to a role in Se
864 transport or homeostasis, but no evidence was found to support this hypothesis (Olson et
865 al., 2010).

866 Glutathione peroxidase 4, that is partly localized in the intermembrane space of the
867 mitochondria (Chen et al., 2002; Orrenius et al., 2007; Savaskan et al., 2007), is
868 considered a critical defense enzyme in protecting membranes against oxidative stress.
869 The critical role of GPX4 was well-defined in GX4-knockout animals, in which
870 embryonic lethality was demonstrated, whereas GPX1-knockout mice are fully viable.
871 Additionally, cell lines heterozygous for GPX4 are markedly sensitive to inducers of
872 oxidative stress, as compared with cell lines derived from wild-type control littermates
873 (Yant et al., 2003).

874 Over the last couple of years, it has become clear that GPX4 is far more than an
875 antioxidant device in cells and tissues, since it regulates a variety of cellular processes,
876 such as polyunsaturated fatty acid metabolism and cellular life and death decisions, and
877 contributes to sperm development at various steps (Conrad et al., 2007).

878

879 **2.5 Specific aspects of ROS and antioxidants in ovulation and early pregnancy**

880

881 **2.5.1 Ovulation**

882 Over the last years, evidences have been accumulated to suggest that ROS and
883 antioxidant enzymes are important for an adequate function of the mammalian
884 reproductive system (Al-Gubory et al., 2010).

885 Within the ovary, ROS are produced as physiological by-products of steroid-driven
886 metabolism, during the follicular and luteal phases (Gonzalez-Fernandez et al., 2005).
887 These metabolites affect follicular growth and development and, thereafter, contribute to
888 oocyte maturation and follicular wall rupture. Additionally, ROS are produced within
889 follicles, especially during the ovulatory process (Paszkowski et al., 1995), and in high
890 levels they are believed to cause poor oocyte quality (Demeestere et al., 2005).
891 The mechanisms that the oocyte uses to alleviate oxidative stress are very
892 complex, including many targets of regulation (Whitaker and Knight, 2008). It has been
893 shown that cumulus cells play a major role in the antioxidant defense of oocyte using
894 GPX and catalase enzymatic activities (Combelles et al., 2009); therefore, it is apparent
895 that no single antioxidant is able to alleviate oxidative stress in the maturing pig oocytes.
896 The rupture of the follicular wall during ovulation can be modeled as a short
897 inflammatory process and, as so, an increased oxidative pressure is induced (Sugino,
898 2005) (Figure 8).
899

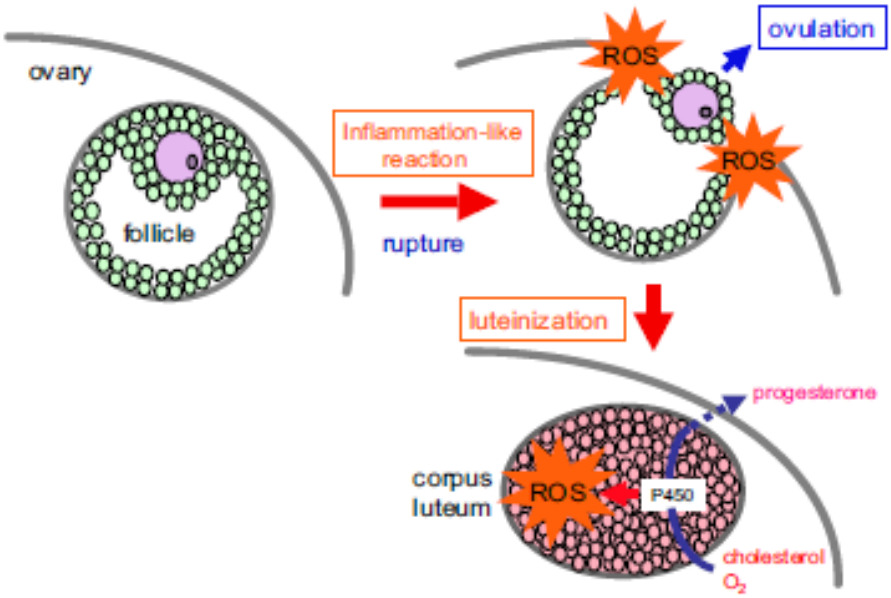


Figure 8 - Reactive oxygen species production in the ovarian follicle (Adapted from Fujii et al., 2005).

Even after ovulation, ROS are still produced by macrophages and neutrophils residing in both follicles and CL (Nakamura et al., 1999). In luteal cells, high levels of ROS have been correlated with decreased P_4 production and cell death (Aten et al., 1992). To counteract the adverse effects of ROS, SOD is present in growing and ovulated follicles, as well as, in the granulosa membrane of Graafian follicles. In the nucleus of the oocyte and in oviductal fluids, high concentrations of GSH and GSH reductase are found and possibly represent the major source of redox potential (Perreault et al., 1988; Yoshida et al., 1993). Additionally, cyclic changes in SOD and GPX levels during the reproductive cycle suggest a role of these antioxidant enzymes in the regulation of follicular development, ovulation, and/or luteal functions (Laloraya et al., 1989a; Lapointe et al., 2005; Fortier et al., 2012).

In swine, limited information is available on biochemical mechanisms and oxidative stress pathways interfering with fertility and embryo development, as well as, how the dietary antioxidant supplements can modulate these processes.

2.5.2 Early pregnancy

Pregnancy is a period of changes in dam's physiology and requirements due to the increased metabolism (Pipkin, 2007). The establishment of pregnancy is promoted by a network of signalling molecules that mediate the cell-to-cell communication between maternal and embryonic tissues (Al-Gubory et al., 2010). Micronutrients affect this process by regulating hormones, growth factors and cell signalling pathways that interfere with both nutrient uptake by the conceptus and the environment in which pre-natal development proceeds (Ashworth and Antipatis, 2001). Additionally, the activity of some enzymes may be controlled to assure a correct balance between metabolites. For example, Levonen et al. (2000) showed that the activity of cystathionine γ-lyase in human fetal liver tissue was undetectable whereas the mRNA is expressed, suggesting a possible post-transcriptional regulation. The activity of this enzyme was detected only in adults.

High levels of oxidants such as O_2^- are produced in the uterus of pregnant animals, with beneficial and detrimental effects depending on the period and level of production (Laloraya et al., 1989a, b). Indeed, increased ROS production in granulosa cells has deleterious effects on embryo quality and, consequently, on implantation rate.

Nutritional and environmental factors play a major role in programming the susceptibility of the offspring to oxidative stress and related disorders. Oxidative stress

programming may operate either directly through the modulation of gene expression or indirectly through the adverse effects of oxidized molecules, such as lipids and proteins, at critical developmental periods (Luo et al., 2006). Considering that the follicular fluid in which the oocyte matures is a product of the transfer of blood constituents (Fortune, 1994), the oocytes are exposed to similar nutritional levels as the dam and may build-up reserves that will be consumed during early embryo development. In fact, Yoshida et al. (1993) showed that the amount of GSH in the porcine oocyte exceeds the needs for fertilization but this level continuously decreases and reaches a nadir at the blastocyst stage. Apart of that, at this stage the embryo is nourished by the uterine fluids that may not reflect maternal blood levels for all nutrients. Therefore, insufficient supplies of nutrients such as essential vitamins and micronutrients result in a state of biological competition between dam and conceptuses, which is detrimental to the health status of both (King, 2003). In fact, the reduced specific antioxidant activities triggered by deficient supply of micronutrients such as copper, zinc, manganese, and Se is associated with poor pregnancy outcomes, including fetal growth restriction (Fall et al., 2003).

The pre-implantation embryo develops under hypoxic and even almost anaerobic conditions (Fischer and Bavister, 1993), however, the embryo itself is a source of ROS. Yoshida et al. (1993) showed that at the blastocyst stage endogenous ROS are produced at their highest level. According to Gardiner and Reed (1994), hydrogen peroxide in mouse blastocoel fluid causes apoptosis of pre-trophectodermal cells of the blastocyst, whereas the inner-cell-mass cells, destined to become the embryo, are protected from the toxic effects of hydrogen peroxide. Therefore, these low GSH and high ROS levels are

959 possibly required for normal differentiation (Parchment, 1993), in which ROS may act as
960 an important regulatory system for apoptosis in the mouse blastocyst (Pierce et al., 1991).
961 Germ cells are more susceptible to ROS and oxidative stress than somatic cells (Nasr-
962 Esfahani et al., 1990; Jancar et al., 2007), and therefore, H_2O_2 production and
963 antioxidants systems, such as GSH-dependent antioxidant mechanisms, must be strictly
964 regulated in the developing embryo (Pierce et al., 1991). In fact, embryonic GSH status
965 changes dramatically during development (Gardiner and Reed, 1994), with GSH
966 synthesis and turnover increasing between the two-cell and blastocyst stages. These
967 metabolic changes are important for cellular signalling pathways involved in
968 proliferation, differentiation, and apoptosis (Dennery, 2007).
969 The pre- and post-placentation environments provide different challenges to the
970 embryo. The rise in O_2 tension brought by placentation is associated with higher
971 expression of ROS markers (Jauniaux et al., 2000) and has been related with inhibition of
972 embryo development (Ufer and Wang, 2011). Indeed, the peri-placentation period in pigs
973 is associated with increased loss of embryo-attachment sites (Gonzalez-Añover et al.,
974 2011). Therefore, these detrimental metabolic by-products must be neutralized locally by
975 antioxidants such as the GPX system.
976 Consequently, understanding the control of the expression of mRNA encoding key
977 antioxidant enzymes and the regulation of their activity, in both maternal and embryonic
978 tissues, is of ultimate importance to improve pre- and post-implantation embryo
979 development. Surprising, little is known about the gene expression and activity of GPX-
980 related enzymes during embryogenesis.

This PhD research program aimed to address the importance of controlling the antioxidative GPX system and more specifically to determine the impact of maternal MSe and OSe supplementation added or not with pyridoxine on the whole genome of the porcine embryo and access the importance of adequate pyridoxine supplementation to the flow of Se towards the antioxidant system, especially the enzyme GPX, and analyse the potential benefits for gilts reproductive performance and embryos development.

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4. OBJECTIVES

4.1 General objective

Measure the homeostasis of selenium and glutathione peroxidase activity in gilts and conceptuses, and its effects on ovarian function and early embryo development in gilts supplemented with organic or inorganic selenium added or not with pyridoxine.

4.2 Specific objectives

Accessing the importance of adequate pyridoxine nutrition to the flow of organic selenium towards the antioxidant system and its impact on the hormonal profile and reproductive performance in gilts.

Determine the impact of maternal Se and B_6 supplementation on the whole genome of the porcine expanded blastocyst and investigate biological processes and key enzymes related to embryo Se metabolism.

Improving the flow of selenium to the glutathione peroxidase system and analyse the potential benefits for gilts reproductive performance and embryos development.

5. PAPERS FOR PUBLISHING

The importance of pyridoxine (vitamin B_6) for the impact of the dietary selenium sources on the antioxidant system, hormonal profile and ovulation rate in gilts[1]

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ABSTRACT: This study aimed to determine the effects of pyridoxine (B_6) combined with dietary selenium (Se) on the antioxidant system, estrus hormone profile, and ovulation rate in gilts. Forty-five gilts were randomly assigned to one of the 5 experimental diets (n = 9/group): 1) basal diet (0.2 and 1.7 mg/kg of natural Se and pyridoxine, respectively) (CONT); 2) basal diet + 0.3 mg/kg of Na-selenite ($MSeB_60$); 3) diet 2 + 10 mg/kg of HCl-B_6 ($MSeB_610$); 4) basal diet + 0.3 mg/kg of Se-enriched yeast ($OSeB_60$); and 5) diet 4 + 10 mg/kg of HCl-B_6 ($OSeB_610$). Blood samples were collected on the d after each onset of estrus and at slaughter (long-term profiles), and daily from d -4 to d +3 of the fourth estrus (peri-estrus profiles). At slaughter (d 3 after the fourth estrus), liver, kidneys, and ovaries were collected, and ovulation rate assessed. For long-term profiles, CONT had lower blood Se than Se-supplemented gilts ($P < 0.01$) and OSe was higher than MSe ($P < 0.01$). Lower erythrocyte pyridoxal-5-phosphate (P-5-P) concentration was found in B_60 than B_610 gilts ($P < 0.01$). No treatment effect ($P > 0.19$) was observed on blood Se-dependent glutathione peroxidase (GPX) activity, plasma vitamin E, ferric reducing antioxidant power, and total antioxidant capacity. For peri-estrus profiles, treatment effects on blood Se and plasma P-5-P were similar to those for long-term profiles. Blood GPX activity was higher in Se-supplemented than CONT gilts ($P < 0.05$) and MSe was higher than OSe ($P < 0.01$). The highest LH surge and ovulation rate were observed in $OSeB_610$ gilts (Se source X B_6 level, $P < 0.05$ and $P = 0.09$, respectively). In liver, CONT had lower Se concentration than Se-supplemented gilts (Se level, $P < 0.01$). For liver and kidney, OSe had higher Se concentration than MSe gilts (Se source, $P < 0.01$). Gene expression of *GPX1*, *GPX3*, *GPX4*, and selenocysteine lyase in liver were greater in Se-supplemented gilts than in CONT ($P < 0.03$), whereas this effect was limited to *GPX1*, *GPX3*, and selenocysteine lyase in kidney ($P = 0.07$). All these genes were found to be highly expressed in $OSeB_610$ gilts (Se source X B_6 level, $P < 0.05$). In conclusion, the Se status and GPX activity of gilts are significantly impacted by both Se level and source during the peri-estrus period. These results suggest that dietary B_6 modulates the metabolic pathway of OSe towards activation of the GPX system, and their combined effect may be involved in the ovarian function leading to optimal ovulation conditions.

Key words: gilt, glutathione peroxidase, ovulation, pyridoxine, selenium, sex hormones

INTRODUCTION

During the peri-estrus period, cellular energy metabolism of ovary is enhanced in response to hormone production and ovulation and it generates reactive oxygen species (**ROS**) as metabolic by-products (Agarwal et al., 2005). Excessive ROS production promotes oxidative stress conditions which have been linked to many adverse reproductive outcomes (Al-Gubory et al., 2010). It is generally known that ROS toxicity is controlled by a complex network of non-enzymatic (notably vitamins E and C) and enzymatic antioxidants, including the selenium-dependent glutathione peroxidase (**GPX**) system. A constant and adequate antioxidant status brought by the positive action of selenium (**Se**) on the GPX system is critical for an optimal follicular development (Abedelahi et al., 2010). This could be particularly important for modern hyperprolific lines of sows where the high ovulation rate has been associated with a poor quality of supplemental oocytes (Driancourt et al., 1998).

The metabolic control of GPX was observed recently in pigs during the peri-estrus period (Fortier et al., 2012). The blood GPX activity dropped shortly after ovulation in control gilts whereas it was maintained and enhanced throughout the peri-estrus period by dietary supplementation with inorganic (**MSe**) and organic Se (**OSe**), respectively. Several reactions of the transsulfuration pathway, which is involved in the fate of OSe for the control of the GPX system, are dependent of vitamin B_6 (Yasumoto et al., 1979) whereas MSe short-circuits the transsulfuration pathway and the regulation of GPX by redox changes (Yasumoto et al., 1979; Johansson et al., 2005).

As the requirements of this vitamin for reproducing pigs have never been established (Matte and Lauridsen, 2013), the present experiment aimed to determine the importance of the interaction between vitamin B_6 and Se metabolisms for an adequate flow of Se towards the GPX system in response to the oxidative stress experienced by gilts during the peri-estrus period.

MATERALS AND METHODS

Animals were used and cared for in accordance with the Canadian Council on Animal Care (2009).

Animals and treatments

Forty-five Yorshire-Landrace gilts were selected for this study at 92.3 ± 3.2 kg BW and 135 to 170 d of age. They were grouped in pens (1.5 x 2.5 m per animal, half-slatted concrete flooring) of 6 to 7 animals until the first estrus was detected. For at least 14 d, they were fed *ad libitum* a basal breeding / gestation diet (Table 1) without Se and pyridoxine (**B_6**) supplements but in excess of the recommended NRC (1998) levels for all other nutrients. From the first estrus, the daily allowance was limited to 2.8 kg. Estrus detection was done by introducing a young boar (8 to 12 months of age) into the pen once daily (10 min) for the first 3 estruses and twice daily (10 min each; between 08:00 and 09:00 h and from 16:00 to 17:00 h) for the fourth estrus. From the first estrus, gilts were placed into individual stalls (0.6 x 2.2 m, half-slatted concrete flooring) and were randomly assigned (according to their BW and blood concentrations of Se) to one of the 5 experimental diets: 1) basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively (Table 1), top-dressed with 50 g of ground corn without supplemental Se or pyridoxine (**CONT**, n = 9); 2) basal diet top-dressed with 50 g of ground corn with supplemental Se, providing an equivalent of 0.3 mg/kg of feed of MSe as sodium selenite (214485 Sigma-Aldrich, St-Louis, MO, USA), and without pyridoxine (**MSe$B_6$0**, n = 9); 3) basal diet top-dressed with 50 g of ground corn with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of MSe as sodium selenite, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA) (**MSe$B_6$10**, n = 9); 4) basal diet top-dressed with 50 g of ground corn with supplemental Se, providing an equivalent of 0.3 mg/kg of feed of OSe as Se-enriched yeast (Alltech, Lexington, KY,USA), and without pyridoxine (**OSe$B_6$0**, n = 9); and 5) basal diet top-dressed with 50 g of ground corn with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of OSe as Se-enriched yeast, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine (**OSe$B_6$10**, n = 9). Gilts were sacrificed 3 d after the fourth estrus. Average BW was 125.8 ± 2.8 kg and 156.1 ± 2.8 kg at the beginning of treatment and the end of the experiment, respectively.

Sampling

Blood samples were collected in EDTA-containing tubes (10 mL; Becton Dickinson and Co., Rutherford, NJ) from the jugular vein by venipuncture on all gilts at arrival to the research Centre, on the d after each onset of estrus, and at slaughter (long-term profiles). On d 12 after the third estrus, all gilts were cannulated at the jugular vein by a nonsurgical technique described by Matte (1999). Blood samples were then collected daily from d -4 to d +3 of the fourth onset of estrus (peri-estrus profiles). At slaughter, the reproductive tract, liver, and kidneys were collected from all gilts (9 per treatment). Liver, uterus, and ovary weights were recorded. Samples of liver and kidney were then dissected and immediately frozen in liquid nitrogen. The left horn of the reproductive tract was flushed with 20 mL of PBS and the liquid was collected (uterine flush) and frozen at - 20 °C. The ovulation rate (number of corpora lutea (**CL**)) and the total length of both uterine horn was recorded.

Blood samples were stored at - 20°C for determination of Se and hemoglobin concentrations and at - 80°C for measurement of GPX activity. Hematocrit was measured in fresh blood. After centrifugation of blood at 1800 x g for 12 min at 4ºC, plasma was separated into aliquots and stored at - 20ºC for vitamin E, luteinizing hormone (**LH)**, estradiol ($\mathbf{E_2}$), ferrous reducing antioxidant power (**FRAP**), total antioxidant capacity (**TAC**), and pyridoxal-5-phosphate (**P-5-P**) (peri-estrus profiles) analyses whereas erythrocytes were stored at - 20ºC for P-5-P analyses (log-term profiles).

Concentration of Se, activity of GPX, and gene expression of different members of the GPX family (*GPX1, 2, 3* and *4*) and Se-cysteine lyase (***SCLY***) were measured in liver and kidneys samples that were stored at - 80°C. Uterine flush was stored at - 20ºC for determination of Se and P-5-P concentrations.

Laboratory analysis

Measurements of Se were performed using a fluorimetric method adapted by Giguère et al. (2005) from the technique of Sheehan and Gao (1990). Pyridoxal-5-phosphate was determined using a fluorimetric method adapted by Matte et al. (1997)

from the technique of Srivastava and Beutler (1973). GPX activity was determined in blood, liver, and kidney using a spectrophotometric method described by Gunzler and Flohé (1985). Activities are reported as milliunit (**mU**) per mg of protein or hemoglobin (blood samples) with 1 unit equal to 1 µmol of NADPH oxidized per minute.

Hemoglobin was measured by colorimetry using Drabkin's solution (Manet, 1969). Measurements of plasma vitamin E (α-tocopherol) were performed by HPLC according to a modification of Audet et al. (2004) of the method described by Bieri et al. (1979) and Driskell et al. (1982). The antioxidant power of blood plasma was measured by FRAP, an indicator of total antioxidant potential defined as the ability of plasma antioxidants to reduce added ferric ions (Benzie and Strain, 1999) and by TAC, an indicator of total antioxidant capacity (Erel, 2004). Plasma LH and E_2 were measured using the method of Kingsbury and Rawlings (1993) as described by Fortier et al. (2012).

For gene expression measurements on *GPX1*, *GPX2*, *GPX3*, *GPX4*, and *SCLY*, approximately 100 mg of liver or kidney tissue sample was homogenized in TRIzol® reagent (Life Technologies, Grand Island, NY), and total RNA was extracted according to the manufacturer's instructions. Total RNA was then treated with DNAse I (Life Technologies) to remove contaminating DNA. First-strand complementary DNA was synthesized using oligo $dT_{13–18}$ primers and the Superscript II pre-amplification system (Life Technologies) following the manufacturer's instructions. Primers were designed using the Primer Express software 3.0 (PE Applied BioSystems, Foster City, CA). Quantitative real-time PCR analyses were performed in a 10-µL reaction volume consisting of forward and reverse primers (Table 2), 5 µL of Power SYBRGreen Master Mix (PE Applied BioSystems), 3 µL of 15 x diluted cDNA, and 0.05 µL of Uracil N-glycosylase AmpErase (PE Applied BioSystems). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

The PCR amplifications were performed on an Applied Biosystems 7500 Fast Real-Time PCR System (PE Applied BioSystems) in triplicate, and standard curves were established in duplicate for each gene. Standard curves were composed of serial dilutions of complementary DNA pools from the same tissue and were used to obtain the relative quantification of mRNA using the standard curve method (Applied Biosystems, User Bulletin #2). Five reference genes (**RG**) were amplified in order to find the best RG to be

used for normalization of relative quantification values. These RG are peptidylprolyl isomerase A (***PPIA***), glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), actin beta (***ACTB***), ubiquitin C (***UBC***), and hypoxanthine phosphoribosyltransferase 1 (***HPRT1***). The NormFinder algorithm, which finds the optimum RG out of a group of different reference genes, was used to determine which RG was the least affected by the treatments (Andersen et al., 2004). The least affected RG were *PPIA* and *HPRT1* in the liver and *PPIA* and *UBC* in the kidney and their average amount were used as a normalisation factor (**NF**). The specificity of the amplified fragments was verified by melting curve analysis (dissociation curve).

For each experimental sample, the amount of the studied genes relative to the appropriate RG mRNA was determined from their respective standard curves. Relative quantity ratios were obtained by dividing the relative quantity units of the studied genes by the respective NF. Mean values from triplicates were used to perform the statistical analyses.

Statistical analysis

Data were analyzed using the SAS procedure for mixed models (SAS Inst. Inc., Cary, NC; Littell et al., 1996) according to a randomized arrangement of treatments in blocks with the 5 dietary treatments in the experimental period as the main independent variables. The model was: $Y_{ijk} = \mu + J_i + W_j + (JW)_{ij} + e_{ijk}$, where Y_{ijk} = dependent variable, μ = general mean, J_i = Se treatments, $Wj = B_6$ treatments, $(JW)_{ij}$ = interaction Se X B_6, and e_{ijk} = residual error. The gilt was considered as the experimental unit. The residual error term was used to test the treatment effects. For the analyses of hormones and other components, sampling times were added to the model as a second factor and were analyzed using repeated option of the MIXED procedure of SAS. Sampling times were considered during the whole experiment (3 samples for long-term profiles) or during the peri-ovulatory period of the fourth estrus (8 samples for the peri-estrus profiles from d -4 to d +3, with d 0 corresponding to physiological estrus as identified by the LH surge). When treatment effects or treatment X time interaction were significant, priori comparisons were done according to the factorial arrangement (Se source, B_6 level, and

Se source X B_6 level interaction) with an additional comparison of all the 4 treatments versus CONT (Se level). Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$, and all results are expressed as adjusted means ± SEM.

RESULTS AND DISCUSSION

Long-term effects of dietary treatments on Se- and B_6-related metabolites

No treatment effect was observed on blood hemoglobin and hematocrit concentrations ($P = 0.29$), but there was a decrease of 15.2 % and 12.2 %, respectively ($P < 0.01$) between the third and fourth estrus.

This time effect is likely due to differences in blood sampling procedures, which were done through the jugular vein catheter for the fourth estrus and by venipuncture after snare restraint for the first, second, and third estrus. It is known that snare restraint procedure induces, within 2 minutes, an elevation of hemoglobin and hematocrit due to release of red blood cells from storage in different organs (Dubreuil et al., 1993). As Se and vitamin B_6 are more concentrated in red blood cells than in plasma (Bender, 2003; Giguère et al., 2005), this might likely interfere with the interpretation of sample values collected for long-term (venipuncture) and peri-estrus (jugular vein catheter) profiles. As the results are repeated measurements according to estrus, data from the first 3 estruses were selected for the evaluation of long-term effects whereas those from the fourth estrus were used to evaluate the peri-estrus effects.

Blood Se concentrations were lower in CONT than in Se-supplemented gilts (Se level, $P < 0.01$). From the first to the third estrus, blood Se levels decreased by 5.5 % for CONT and increased by 3.9 % for MSe and by 9.3 % for OSe gilts (treatment X time interaction, $P < 0.01$; Figure 1). Blood Se concentrations were greater in OSe gilts than in MSe, but only for the third estrus (Se source, $P < 0.01$). Such result is in accordance with Quesnel et al. (2008) showing that the effect of Se sources on blood Se content depends on the duration of supplementation. For the CONT group, no animal exhibit any signs of Se deficiency but the present results suggest that they were developing a negative Se

balance in spite of a natural level of Se (0.2 mg/kg) in the basal gestation diet which is higher than the NRC (1998, 2012) recommendation.

The Se source effect on blood Se concentration of gilts is consistent with previous reports (Svoboda et al., 2008; Fortier et al., 2012). The absorbed MSe (selenite) is quickly transformed into selenide and then converted by selenophosphates into functional selenoproteins containing selenocysteine instead of being actively stored in organs (Windisch, 2002). Selenomethionine, the main source of OSe, is either metabolized directly to give reactive forms of Se or stored as selenomethionine in substitution of sulfur methionine during protein synthesis (Schrauzer, 2003), thus becoming a constituent of tissue proteins such as haemoglobin in blood erythrocytes or albumin in plasma (Butler et al., 1991; Schrauzer, 2000). In terms of dietary B_6 supplementation, no effect was observed on blood Se concentration (B_6 level, $P = 0.49$).

For long-term profiles, erythrocyte P-5-P was chosen as an indicator of vitamin B_6 status. In pig erythrocytes, P-5-P is over six times more concentrated as compared to plasma and its storage in these cells is saturable (Matte et al., 2001) probably because it is bound to specific enzymes or proteins (Bender, 2003). For the whole experimental period, lower erythrocyte P-5-P concentrations were found in $B_6$0 than in $B_6$10 gilts (B_6 level, $P < 0.01$). From the first to the third estrus, erythrocyte P-5-P concentrations decreased by 15.1 % and 10.4 % for CONT and $B_6$0 groups, respectively, and increased by 29.5 % for $B_6$10 group (treatment X time interaction, $P < 0.01$). Average pre-treatment values were 4.96 ± 0.27, 5.21 ± 0.27, and 4.81 ± 0.27 uM, and third estrus values were 4.21 ± 0.29, 4.67 ± 0.27, and 6.23 ± 0.28 uM for CONT, $B_6$0 and $B_6$10 groups, respectively.

The decrease of erythrocyte P-5-P concentrations observed in CONT and $B_6$0 groups suggest a negative B_6 balance in these gilts. Such response was observed in spite of a dietary vitamin B_6 concentration of 2.3 mg/kg in the basal diet (analytical value expressed as pyridoxine equivalent, i.e. sum of pyridoxamine, pyridoxal, and pyridoxine in the mix feed), a level which exceeded the NRC (1998, 2012) recommendation of 1 mg/kg. There was no overall effect ($P = 0.86$) of Se supplementation on erythrocyte P-5-P concentrations.

Long-term effects of dietary treatments on the antioxidative status

There was no treatment effect on GPX activity in blood ($P = 0.19$) with average values of 127.72 ± 6.21, 131.45 ± 6.21, and 130.58 ± 6.21 mU/mg Hb for the first, second, and third estrus, respectively. In the literature, responses of blood GPX activity to Se sources are rather inconsistent. Some authors have shown higher GPX activity in blood and plasma of gilts supplemented with MSe (Kim and Mahan, 2001; Fortier et al., 2012), whereas others found no differences between OSe and MSe either in blood, plasma, or serum (Acda and Chae, 2002; Mahan and Peters, 2004; Yoon and McMillan, 2006; Svoboda et al., 2008). This inconsistency related to GPX activity might be due to variations in the selenomethionine content of sources of Se-enriched yeast used in the different studies (Zhan et al., 2010). It was also shown that the regulation of GPX activity by dietary Se is strongly influenced by the initial Se status of the animals. Indeed, animals fed a low Se diet before supplementation showed a higher requirement for this mineral and a greater response of the GPX system when adequate dietary Se levels were provided (Mahan et al., 1999).

There was no treatment effect on vitamin E, FRAP, and TAC plasma levels ($P > 0.19$) from the first to the third estrus. Concentrations averaged 2.06 ± 0.16 ug/mL, 136.71 ± 5.13 uM, and 0.24 ± 0.02 mmol, for α-tocopherol, FRAP, and TAC, respectively. Along with GPX activity in blood, these results suggest that the overall antioxidant status of the gilts was not affected by Se and B_6 supplementations on a long-term basis.

Effects of dietary treatments on Se- and B_6-related metabolites during the peri-estrus period

During the peri-estrus period, CONT had lower blood Se concentration than Se-supplemented gilts (Se level, $P < 0.01$; Figure 2), and OSe gilts had higher concentrations than MSe (Se source, $P < 0.01$). From d -4 to d -1 prior to ovulation, blood Se concentration decreased in all treatments. However, from d -4 to d 3, Se concentration increased by 3.0 % and 1.0 % for OSe and MSe, respectively, and decreased by 1.9 % for CONT (treatment X time interaction, $P < 0.05$) (Figure 2). Although small, those effects

occurred within a short period of one week. For CONT gilts, this is in continuum with the long-term developing Se negative balance observed during the preceding 3 estruses where the basal dietary provision of Se was apparently not adequate to cover metabolic needs for this trace element. As for the long-term profile, no B_6 treatment effect was observed on blood Se concentration (B_6 level, $P > 0.62$).

In contrast to long-term profiles using erythrocytes, plasma P-5-P was chosen as an indicator of systemic response to an eventual transient mobilization and/or utilization of P-5-P during the peri-estrus period. Plasma P-5-P concentrations were affected by the peri-estral time ($P \leq 0.01$), with maximum values at d -3 and minimum values at d 2 (1.93 ± 0.07 uM and 1.79 ± 0.07 uM, respectively). For the whole peri-estrus period, values were higher in $B_6$10 than in $B_6$0 gilts (2.00 ± 0.07 and 1.75 ± 0.07 uM, respectively; B_6 level, $P < 0.01$), but no treatment X time interaction was observed ($P = 0.93$). OSe gilts tended to have higher plasma P-5-P concentrations than MSe gilts (Se source, $P = 0.09$). As this last tendency have occurred whatever the B_6 status of the animals, it may reflect an increased mobilization of P-5-P in OSe gilts during the peri-estrus period.

Effects of dietary treatments on the* GPX *status during the peri-estrus period

During the peri-estrus period, Se-supplemented groups had higher blood GPX activity than CONT (Se level, $P < 0.01$) and MSe was higher than OSe (Se source, $P < 0.01$). Although this response is different from the long-term effects, both effects emphasize the fact that the sole measure of total blood Se could not accurately reflect the amount of Se that is directly available for the activity of selenoproteins as reported by Thomson (2004). From d -4 to d -1, GPX activity decreased by 3.7 % and 1.7 % for CONT and OSe, respectively, and increased by 3.4 % for MSe, but from d -1 to d 3 these activities increased by 4.4 % for OSe and decreased by 4.0 % and 2.5 % for CONT and MSe, respectively (treatment X time interaction, $P < 0.05$; Figure 3). Dietary Se treatment effects were from sources ($P < 0.01$) on d -4 and d -3, and from both sources and levels from d -2 until d 3 ($P < 0.05$).

The constant decrease of blood GPX activity during the peri-estrus period in CONT gilts, which was also observed by Fortier et al. (2012), further suggest that these animals were responding to the oxidative burst occurring around the time of ovulation. During this period, the breakdown of follicle walls and the release of mature ova induces an inflammatory reaction characterized by ROS generation from inflammatory cells like macrophages and neutrophils which are massively recruited at the ovulation site (Richards et al., 2002). Although inflammation processes and ROS production have been shown to be indispensable for ovulation (Shkolnik et al., 2011), they must be tightly regulated because uncontrolled inflammatory response and oxidative stress have also been linked to ovulatory dysfunction (Al-Gubory et al., 2010). Such regulation appeared more apparent in OSe gilts where the peri-estrus profile of GPX activity suggest a shift of this enzyme in response to oxidative pressure of ovulation as also suggested by Fortier et al. (2012).

As indicated by the absence of B_6 effect or interaction with Se treatments, the response of blood GPX activity in OSe gilts was not dependent upon the B_6 status of the animals within the range induced by the present B_6 treatments. This contrasts with previous results in rats showing that the positive actions of selenomethionine on the GPX system are mediated by vitamin B_6 (Yasumoto et al., 1979). However, such effects were observed in vitamin B_6-deficient animals after supplementation with vitamin B_6, whereas in the present study the basal diet exceeded the recommended amount of vitamin B_6.

Impacts of dietary Se and B_6 supplementation on estradiol and luteinizing hormone levels and ovulation rate

There was no treatment effect on plasma E_2 concentrations ($P = 0.83$). Considering the global peri-estrus period, no effect of treatment was found on plasma LH ($P = 0.12$), however, for the LH surge on d 0, values were higher in OSe$B_6$10 gilts than in others (Se source X B_6 level, $P < 0.05$, Figure 4). Such results contrast with those reported by Fortier et al. (2012) where LH was not influenced by sources or levels of dietary Se, whereas peri-estrus profiles of E_2 were lower in MSe than in OSe gilts. It is noteworthy to mention that, in Fortier et al. (2012), all diets were supplemented with 3.0

mg/kg of hydro-chloride pyridoxine. Taken together, these previous and present results suggest that Se and B_6 play a role in the hormonal control of ovulation in pig. The implication of Se in hormone regulation first arises from the general observation that Se was preferentially retained or redistributed to the brain, endocrine and reproductive organs during episodes of Se depletion (Kohrle et al., 2005). In other species, Se stimulates cells proliferation of small follicles and improves their response to the stimulatory effects of gonadotropins (Pappas et al., 2008), whereas the secretion of hypothalamic LH is greater in B_6-supplemented than B_6-deficient animals (Bender, 2003). In view of that previous knowledge, it can be hypothesized that the combined effect of OSe and B_6 improved the secretion of LH and the number of follicles available to grow and ovulate, as observed in the ovulation rate (e.g. number of CL) which tended to be greater in OSe$B_6$10 gilts (Se source X B_6 level, $P = 0.09$) (Table 3). The present absence of treatment effect on peri-estrus E_2 suggests that E_2 production per follicle was smaller in OSe$B_6$10 gilts. Further investigations are required to assess the quality of ova and eventually of embryos derived from these supplementary follicles in order to better characterize the roles of Se and B_6 in the ovulation process in pigs.

Effects of dietary treatments on organ physiology and Se content at slaughter

There was no treatment effect on the length of the uterine horn ($P = 0.74$), as well as on liver ($P = 0.48$), ovary ($P = 0.93$), and uterus weights ($P = 0.76$) (Table 3). In the uterine flush, P-5-P concentrations were similar among treatments ($P = 0.56$) but Se was not detectable (< 10 ng/mL).

In liver, concentrations of Se were lower in CONT than in Se-supplemented gilts (Se level, $P < 0.01$), whereas values were greater in OSe than MSe gilts for both liver and kidneys (Se source, $P \leq 0.01$, Table 4). The Se source effects are in accordance with several previous reports showing greater Se deposition in OSe than MSe in different tissues including liver and kidneys in pigs (Mahan and Kim, 1996; Šobajić et al., 1998; Mahan and Peters, 2004; Mahan et al., 2005; Mateo et al., 2005). However, the difference for Se level effect (or not) in these tissues might be related to peculiar Se metabolism according to organs. In fact, in kidneys, the tissue was enriched in Se only for OSe gilts (Se source, $P < 0.01$, Table 4). Excess MSe absorbed from the digestive tract and not

utilised in selenoprotein synthesis is methylated (Itoh and Suzuki, 1997) and rapidly excreted from the body (Árová et al., 2003). For dietary OSe, selenized amino acids as all other amino acids are almost completely reabsorbed in the kidney capillaries and re-enter the whole body metabolism (Robinson et al., 1997). Indeed, Thomson (1998) found that 82-95 % of the ingested MSe can be found in the urine, whereas the value was estimated at approximately 26 % for OSe, in humans. Taken together, such responses suggest that the higher Se concentrations in kidneys of OSe gilts were related to a higher renal re-uptake of Se in these animals.

Impacts of dietary Se and B_6 supplementation on GPX activity and mRNA expression in liver and kidney

No effect of Se, B_6 or their interaction was observed on GPX activity in liver and kidney ($P > 0.19$, Table 4). However, for gene expression in liver, Se-supplemented groups had higher *GPX1* (60.30 %), *GPX3* (56.14 %), *GPX4* (40.20 %), and *SCLY* (28.37 %) values than CONT (Se level, $P < 0.01$) (Figure 5). In kidney, Se-supplemented groups had higher *GPX1* (89.33 %) and *GPX3* (91.48 %) gene expressions than CONT (Se level, $P < 0.01$), whereas the effect was a tendency for *SCLY* (22.94 %) (Se level, $P = 0.07$), and no treatment effect was observed on *GPX4* ($P = 0.18$) (Figure 6). Moreover, renal *GPX3* values were higher in OSe than in MSe gilts (Se source, $P < 0.01$) and in $B_6$10 than $B_6$0 gilts (B_6 level, $P < 0.05$). For both tissues, the highest gene expression of *GPX1* and *SCLY* were observed in OSe$B_6$10 gilts (Se source X B_6 level, $P < 0.01$ and $P < 0.05$ for liver and kidneys, respectively). No detectable expression of *GPX2* was observed in liver and kidney. These results indicate that *GPX* mRNA expression in liver and kidneys could be modulated by dietary Se in gilts. The observed difference in mRNA expression between the members of the GPX family and the absence of response for *GPX4* in the kidneys might be due to the hierarchy of importance of selenoproteins in the tissues (Brigelius-Flohé and Maiorino, 2013). Indeed, it is well-known that the expression of the various *GPX* is differentially affected by either Se deficiency or supplementation depending of their physiological relevance. For example, it was determined that *GPX4* mRNA expression is only mildly affected by declines in tissular Se content in contrast to *GPX1* (Lei et al., 1995; Brigelius-Flohé and Maiorino, 2013).

A discrepancy between GPX activity and mRNA abundance has been reported before for Se-supplemented animals (Thomson, 2004). It has been shown that the increase in *GPX* mRNA levels precedes the increase in GPX activity that normally occurs with increasing dietary levels of Se in rats. This sequence of responses to dietary Se can be explained by the fact that the Se requirement for maximizing *GPX* mRNA is half the level needed to reach maximal GPX activity (Weiss et al., 1996). For the present study, this suggests that dietary Se supplements were sufficient to induce a *GPX* mRNA response but not to promote GPX activity in liver and kidneys of gilts. The absence of correspondence between *GPX* mRNA levels and activity was also reported for gestating sows that were fed with basal and supplemented levels of 0.05 and 0.15 mg/kg of selenite (Hostetler et al., 2006). In that last case, however, differences between treatments were observed on GPX activity but not on gene expression. This could also be explained, as above, by the fact that *GPX* mRNA levels responded to increasing Se status and reach a plateau before GPX activity. It is generally known that GPX can be regulated by transcriptional, post-transcriptional, translational, or post-translational factors and changes in gene transcription may not be reflected on GPX protein or on its related enzyme activity (Lubos et al., 2011). In an *in vitro* model using human myeloid cell line, it was shown that GPX genes are regulated post-transcriptionally in response to Se availability (Chada et al., 1989). These last authors suggest that the most probable mechanism for the post-transcriptional regulation would be related to co-translational incorporation of selenocysteine into GPX through selenocysteyl-tRNA. This metabolic pathway requires the B_6-dependent oxidation of selenocysteine through SCLY (Yasumoto et al., 1979). The present dual treatment effects on both *GPX's* and *SCLY* gene expressions suggest that the co-translation process towards GPX's synthesis was not limited by post-transcription of SCLY and was in synchrony with the selenocysteine-selenocysteyl pathway.

Conclusions

Both Se levels and sources, but not B_6 levels, affected the Se status of gilts. However, on a long-term basis, these effects on Se status were not reflected on measurements of antioxidative status in blood and GPX activity in tissues.

The peri-estrus period appears to be an adequate model to study treatment responses to oxidative stress conditions using Se homeostasis and variations of blood GPX activity. Although not influenced by B_6, the daily profile of blood GPX activity varied according to levels and sources of Se supplementation.

The complementation of OSe with vitamin B_6 (as pyridoxine) maximized the expression of *GPX* and *SCLY* genes in liver and kidneys collected shortly after estrus, suggesting a critical role of this vitamin for an adequate flow of selenocysteine towards the GPX system in response to oxidative stress pressure induced by this physiological stage in sows.

The combined effects of OSe with B_6 on GPX activity, peri-estrus LH and ovulation rate deserves further investigations particularly in terms of quality of ova and eventually of embryos derived from these supplementary follicles.

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Table 1. Composition of the basal diet (as-fed basis)[1,2].

Ingredients	Amount, %
Corn	52.6
Wheat shorts	20.0
Distillers dried grain with solubles.	10.0
Canola meal	9.7
Soybean hulls	4.0
Limestone	2.0
Salt	0.6
Monocalcium phosphate	0.5
L-Lysine	0.1
Choline	0.1
Feed curb[3]	0.1
Mineral and vitamin premix[4]	0.3

[1]The calculated compositions for ME, CP, lysine, Ca, and P of the basal diet were 2,702 kcal/kg, 14.0, 0.6, 1.0, and 0.6 %, respectively.

[2]The basal Se and pyridoxine content of the diet were 0.2 mg/kg and 1.7 mg/kg, respectively (analytical values determined according to Giguère et al., 2005 and Matte et al., 2001, respectively).

[3]Inhibitor of mold growth

[4]Provided per kilogram of diet: Mn as manganous oxide, 40 mg; Zn as zinc oxide, 150 mg; Fe as ferrous sulfate, 140 mg; Cu as copper sulfate, 21 mg; I as calcium iodate, 2.0 mg; vitamin A, 14,580 IU; vitamin D, 1,500 IU; vitamin E, 44 IU; vitamin K, 2.6 mg; thiamine, 2.7 mg; riboflavin, 4.9 mg; niacin, 31 mg; pantothenic acid, 21 mg; folic acid, 10 mg; biotin, 400 µg; and vitamin B12, 25 µg.

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Table 2. Primer sequences used for real-time PCR amplifications of selected and reference genes.

Genes[1]	Primer sequences (5'→3') [2]	GenBank Accession no.	Product size (nt)	Primer (nM)
GPX1	(F) CGATGCCACTGCCCTCAT	NM_214201	98	300
	(R) GGCCCACCAGGAACTTCTC			300
GPX2	(F) CCAGAAGTGTGACGTGAATGGT	NM_001115136	106	600
	(R) GGATCAGTCATGAGGGAAAAC			600
GPX3	(F) CTCGGAGATTCTGTCCACTCTCA	NM_001115155	90	300
	(R) CCGTTCACGTCCCCTTTCT			300
GPX4	(F) GCTGGCTACAACGTCAAATTTG	NM_214407	100	300
	(R) TCCCCTTGGGCTGGACTT			300
SCLY	(F) ATCGTGGGCCACAAGTTCTATG	FJ860901	106	600
	(R) GCTCTTGTCCACCTCCAAACA			600
ACTB	(F) CATCACCATCGGCAACGA	XM_003124280	128	300
	(R) GGATGTCGACGTCGCACTT			300
GAPDH	(F) CCCCAACGTGTCGGTTGT	NM_001206359	91	300
	(R) CTCGGACGCCTGCTTCAC			300
HPRT1	(F) GACCAGACTTTGTTGGATTTGAAA	NM_001032376	94	300
	(R)CAAACATGATTCAAGTCCCTGAAG			300
PPIA	(F) GCACTGGTGGCAAGTCCAT	NM_214353	71	300
	(R) AGGACCCGTATGCTTCAGGA			300
UBC	(F) GGTGGGATGCAGATCTTCGT	XM_003483411	98	300
	(R) TGGATCTTCGCCTTGACATTC			300

[1] *GPX* = Glutathione peroxidase; *SCLY* = selenocysteine lyase; *ACTB* = actin, beta; GAPDH = glyceraldehyde-3-phosphate; *HPRT1* = hypoxanthine phosphoribosyltransferase 1; PPIA = peptidylpropyl isomerase A; *UBC* = ubiquitin C.

[2] F = forward primer; R = reverse primer.

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Table 3. Tissue measurements at slaughter and P-5-P in uterine flush.

	CL number	Uterine horn length, cm	Liver weight, g	Ovary weight, g	Uterus weight, g	Uterine flush P-5-P, ng/flush
CONT	17.4	206.7	1811.3	11.5	631.1	972.5
$MSeB_60$	16.7	201.6	1743.9	11.0	579.9	1008.6
$MSeB_610$	17.7	191.2	1776.7	11.1	618.9	1307.2
$OSeB_60$	16.9	194.8	1831.2	11.0	567.5	903.9
$OSeB_610$	21.2	212.6	1913.9	12.2	620.9	1148.3
SEM	0.9	12.3	68.4	1.1	41.2	197.8
a priori comparisons						
CONT vs Se-suppl.	0.54	0.63	0.95	0.89	0.46	0.57
MSe vs OSe	0.06	0.56	0.11	0.63	0.90	0.47
B_60 vs B_610	0.01	0.77	0.40	0.57	0.27	0.14
interaction	0.09	0.26	0.72	0.63	0.86	0.88

Shown are LS means.

CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

Table 4. Selenium concentration and GPX activity in tissues, on day 3 after the fourth estrus.

	Liver Se, ng/g	Liver GPX act., mU/g protein	Kidney Se, ng/g	Kidney GPX act., mU/g protein
CONT	665.6	298.8	2546.4	367.0
$MSeB_60$	801.0	307.4	2496.1	370.7
$MSeB_610$	744.9	281.1	2403.9	333.2
$OSeB_60$	968.0	288.1	2694.6	348.6
$OSeB_610$	979.0	289.0	2694.9	336.6
SEM	26.2	13.5	75.7	22.0
a priori interactions				
CONT vs Se-suppl.	< 0.01	0.84	0.75	0.77
MSe vs OSe	< 0.01	0.44	< 0.01	0.46
B_60 vs B_610	0.38	0.29	0.53	0.24
interaction	0.19	0.94	0.53	0.75

Shown are LS means.

CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

Figure caption

Figure 1. Blood selenium concentration (ng/ml) of gilts on each estrus, presented as LS means ± SEM. CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, MSe = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), OSe = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY). Initial concentrations of blood Se (at arrival) were 321.1 ± 7.4, 325.1 ± 7.6, and 326.7 ± 7.4 µg/mL for CONT, MSe, and OSe gilts, respectively (P = 0.86).
CONT < Se-suppl. (Se level, $P < 0.01$); MSe < OSe (Se source, $P < 0.01$); treatment X times interaction ($P < 0.01$).

Figure 2. Blood selenium concentration (ng/ml of blood) of gilts at fourth estrus, shown as LS means ± SEM. Day 0 was the day of behavioural estrus.
CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, MSe = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), OSe = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY).
CONT < Se-suppl. (Se level, $P < 0.01$); OSe > MSe (Se source, $P < 0.01$); treatment X time interaction ($P < 0.05$).

Figure 3. Blood GPX Activity (mU/mg Hb) of gilts at fourth estrus, shown as LS means ± SEM. Day 0 was the day of behavioural estrus.
CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, MSe = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), OSe = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY).
CONT < Se-suppl. (Se level, $P < 0.01$); MSe > OSe (Se source, $P < 0.01$); treatment X time interaction ($P < 0.05$).

Figure 4. Luteinizing hormone (LH) (ng/mL of plasma) of gilts at fourth estrus, shown are LS means ± SEM. Day 0 was the day of physiological estrus and individual LH surge occurred on day 0 or day 1. CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).
No effect on LH profile ($P = 0.12$); highest LH surge for $OSeB_610$ (Se source X B_6 level, $P < 0.05$).

Figure 5. Gene expression of *GPX1*, *GPX3*, *GPX4* and *SCLY* in liver of gilts 3 days after fourth estrus, shown as LS means ± SEM.
CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).
CONT < Se-suppl. (Se level, $P < 0.01$) for *GPX1*, *GPX3*, *GPX4* and *SCLY*; highest *GPX1* and *SCLY* gene expression for $OSeB_610$ (Se source X B_6 level, $P < 0.01$).

Figure 6. Gene expression of *GPX1*, *GPX3*, *GPX4* and *SCLY* on kidney of gilts 3 days after fourth estrus, shown as LS means ± SEM.
CONT = basal diet containing 0.2 mg/kg and 1.7 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg

of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), OSe$B_6$0 = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), OSe$B_6$10 = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

No treatment effect for *GPX4* ($P = 0.18$); CONT < Se-suppl. (Se level, $P \leq 0.01$) for *GPX1* and *GPX3*; CONT < Se-suppl. ($P = 0.07$) for *SCLY*; highest *GPX1* and *SCLY* gene expression for OSe$B_6$10 (Se source X B_6 level, $P < 0.05$); OSe > MSe (Se source, $P < 0.01$) for *GPX3*; $B_6$10 > $B_6$0 (B_6 level, $P < 0.05$) for *GPX3*.

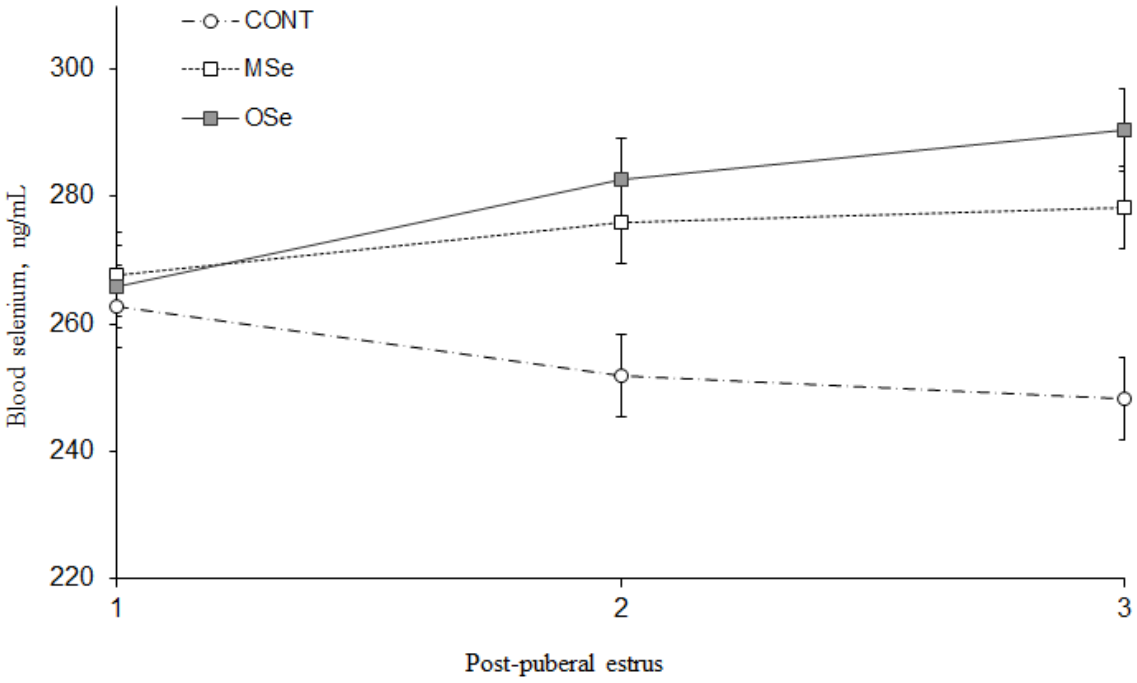

CONT
MSe
OSe
300
280
260
240
220
Blood selenium, ng/mL
1
2
3
Post-puberal estrus

Figure 1

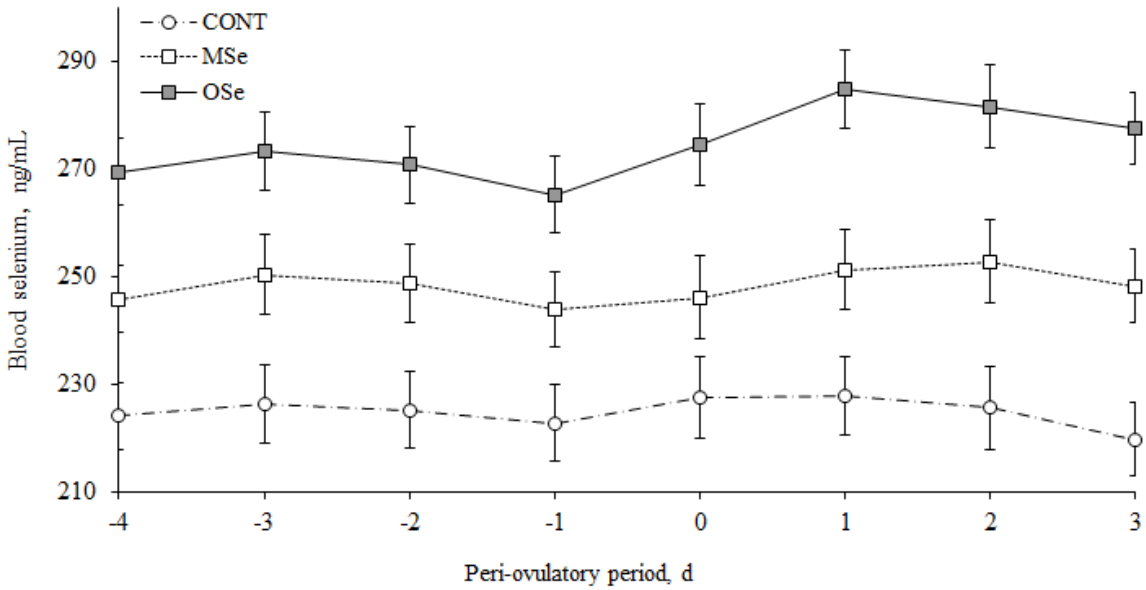
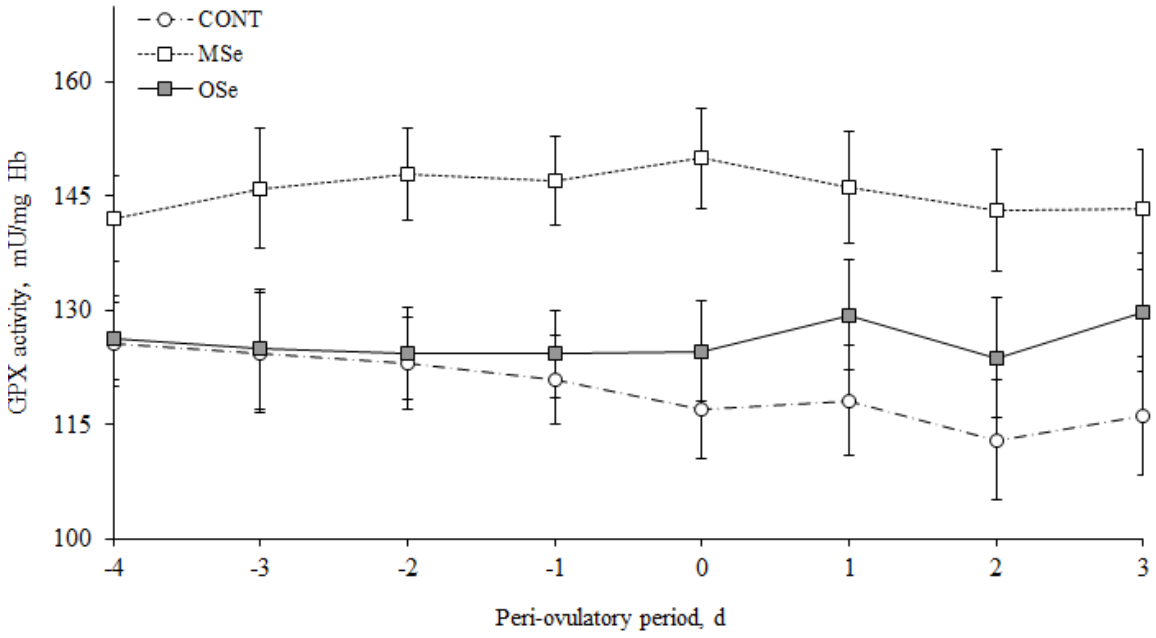

CONT
MSe
OSe
290
270
250
230
210
Blood selenium, ng/mL
-4
-3
-2
-1
0
1
2
3
Peri-ovulatory period, d

Figure 2



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2846 **Figure 3**
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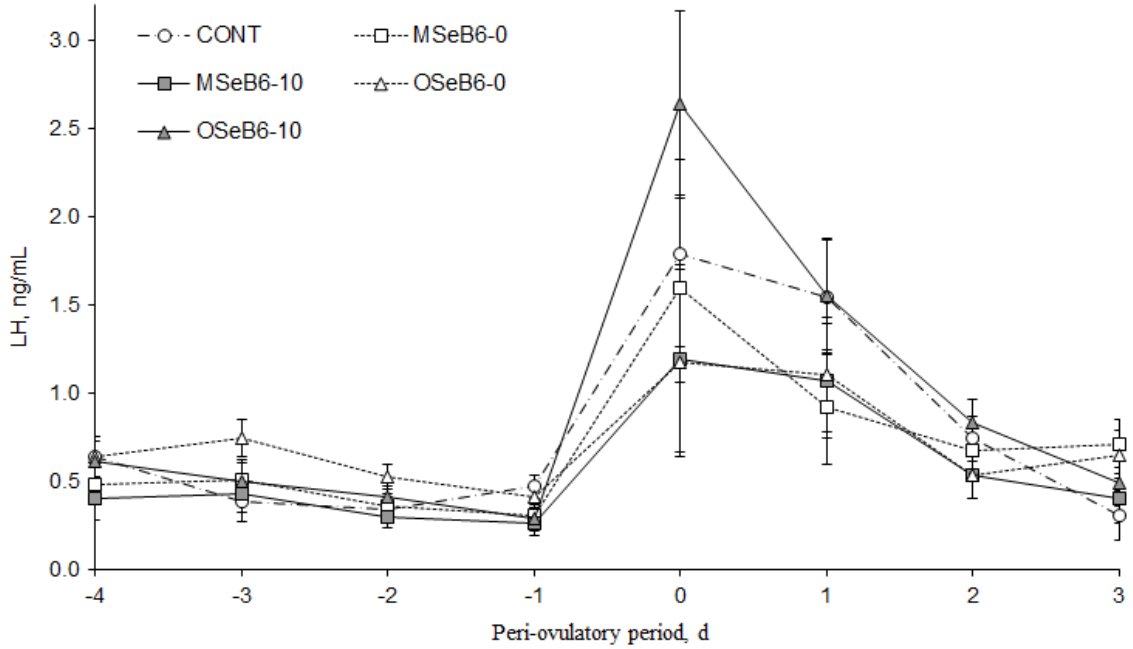

2849
2850 **Figure 4**
2851

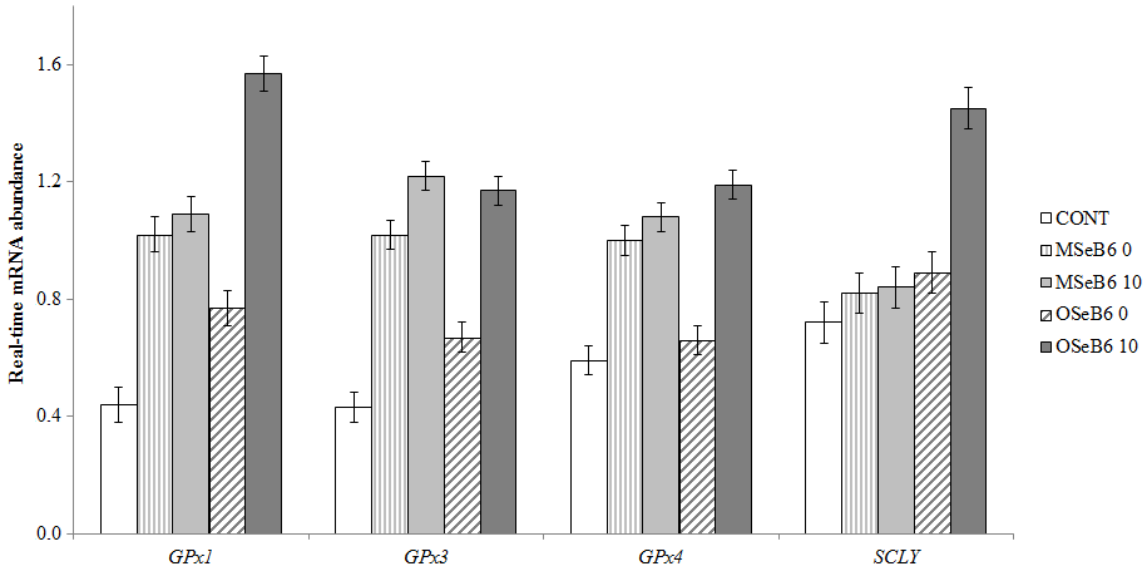
Real-time mRNA abundance
1.6
1.2
0.8
0.4
0.0
GPx1
GPx3
GPx4
SCLY
CONT
MSeB6 0
MSeB6 10
OSeB6 0
OSeB6 10

Figure 5

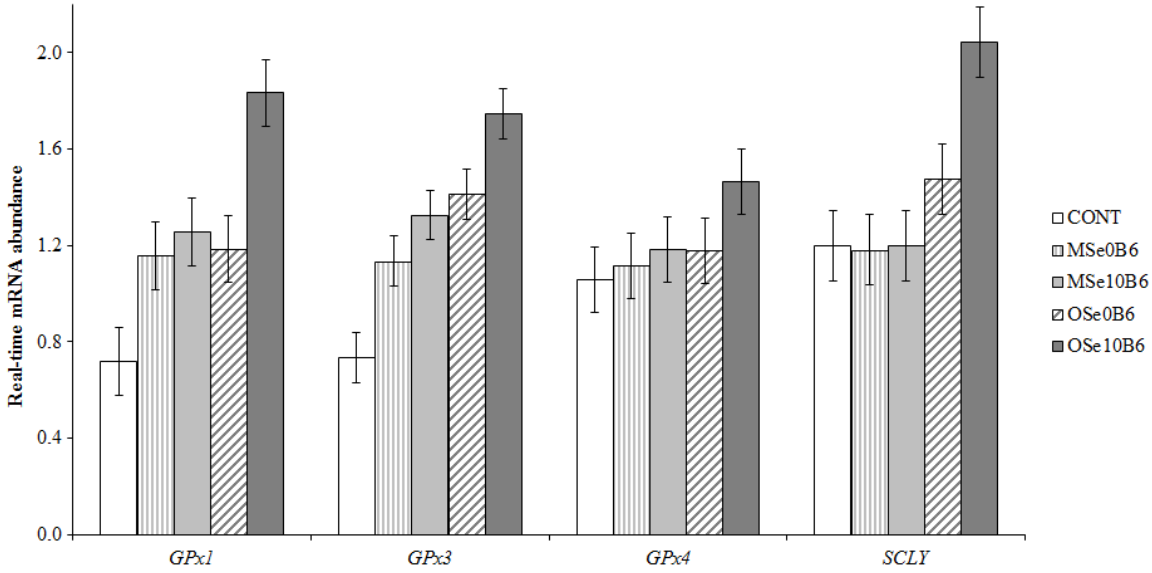
Real-time mRNA abundance
2.0
1.6
1.2
0.8
0.4
0.0
GPx1
GPx3
GPx4
SCLY
CONT
MSe0B6
MSe10B6
OSe0B6
OSe10B6

Figure 6

Title: Gene expression of porcine blastocysts from gilts fed organic or inorganic selenium and pyridoxine

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Short title: Dietary selenium on blastocyst gene expression

ABSTRACT

This study determined how maternal dietary supplementation with pyridoxine combined with different sources of selenium (Se) during pregnancy affected global gene expression of porcine expanded blastocysts. Eighteen gilts were randomly assigned to one of the three experimental diets (n = 6 per treatment): 1) basal diet without supplemental Se or pyridoxine (CONT); 2) CONT + 0.3 mg/kg of Na-selenite, and 10 mg/kg of HCl-pyridoxine ($MSeB_6$10); and 3) CONT + 0.3 mg/kg of Se-enriched yeast, and 10 mg/kg of HCl-pyridoxine ($OSeB_6$10). All gilts were inseminated at their fifth post-pubertal estrus and sacrificed 5 d later for embryo harvesting. A porcine embryo-specific microarray was used to detect differentially gene expression between $MSeB_6$10 vs CONT, $OSeB_6$10 vs CONT and $OSeB_6$10 vs $MSeB_6$10. CONT gilts had lower whole blood Se and erythrocyte pyridoxal-5-P concentrations than supplemented gilts ($P < 0.05$). No treatment effect was observed on blood plasma Se-glutathione peroxidase activity ($P = 0.57$). There were 10, 247 and 96 differentially expressed genes for $MSeB_6$10 vs CONT, $OSeB_6$10 vs CONT and $OSeB_6$10 vs $MSeB_6$10, respectively. No specific biological process was associated to $MSeB_6$10 vs CONT. However, for $OSeB_6$10 vs CONT, up-regulated genes were related to global protein synthesis but not to selenoproteins. The stimulation of some genes related to monooxygenase and thioredoxin families was confirmed by RT-qPCR. In conclusion, $OSeB_6$10 affects porcine expanded blastocysts metabolism more markedly than $MSeB_6$10. Neither Se sources with pyridoxine influenced the Se-glutathione peroxidase metabolic pathway in the porcine expanded blastocysts, but $OSeB_6$10 selectively stimulated genes involved with antioxidant defense.

INTRODUCTION

During the last decades, marked increases in ovulation rate in pigs have been associated with reduced embryo survival (Freking *et al.*, 2007; Patterson *et al.*, 2008), possibly due to the poor quality of oocytes shed by these supplemental follicles (Driancourt *et al.*, 1998).

The process of ovulation induces the production of reactive oxygen species (ROS) in the ovary after the LH surge (Brännström *et al.*, 1993). Peri-estrus oxidative stress has been associated with reduced fertility, and perturbation of oocytes (Guerin *et al.*, 2001) and embryonic development (Ufer and Wang, 2011) in many species. Despite this cytotoxicity, a basal oxidation stress appears necessary for the ovulation process (Van der Hoek *et al.*, 2000) and subsequent embryo development (Orsi and Leese, 2001). Thus, understanding how embryo metabolism regulates the intracellular redox potential is of fundamental importance.

Selenium (Se) is an essential trace element derived from inorganic or organic sources. Organic selenium (OSe), the natural form present in feed, is deposited into proteins as selenomethionine (SeMet) following the methionine metabolism (Schrauzer, 2003), whereas inorganic selenium (MSe), commonly used as a dietary supplement, is readily transformed into selenide and incorporated into some selenoproteins (Windisch, 2002). Roughly half of the selenoproteins confer cellular protection against oxidative stress. Both sources of Se can result in Se-dependent glutathione peroxidase (SeGPX) biosynthesis but the OSe pathway is more complex and responsive to oxidative stress (Gonzalez-Flores *et al.*, 2013). Several reactions of OSe to SeGPX pathway are pyridoxine-dependent (Yasumoto *et al.*, 1979). Roy *et al.* (2011) have shown the

2943 importance of pyridoxine for an adequate flow of OSe towards the SeGPX system in
2944 response to oxidative stress induced during the peri-estrus period in gilts.
2945 Although limited, the information on the effects of sources of supplemental Se for
2946 sows on developing porcine embryos has shown an increased Se transfer of 60 % to
2947 embryos from OSe dams along with an enhanced morphological and physiological
2948 development at 30 d of gestation. This was observed in spite of a lack of effect on
2949 embryo SeGPX activity (Fortier *et al.*, 2012). The metabolic and physiological
2950 mechanisms involved in such responses deserve further investigation. Genomic
2951 approaches such as microarray technologies, which have been developed to study gene
2952 expression of the whole genome of developing mammalian embryos (Niemann *et al.*,
2953 2007), can be powerful tools for that purpose. Recently, a gene expression microarray
2954 specific for porcine embryos was developed and validated (Tsoi *et al.*, 2012), allowing an
2955 efficient analysis of large numbers of different pre-determined transcripts in several
2956 samples.
2957 The present study aimed to determine the effect of maternal OSe or MSe dietary
2958 supplementation combined with pyridoxine on gene expression of the whole genome of
2959 porcine expanded blastocysts (PEB) via microarray analysis. Additionally, biological
2960 processes and some key enzymes/proteins related to embryo Se metabolism, in particular
2961 SeGPX, were also investigated.
2962

2963 **MATERIAL AND METHODS**

2964 *Animals and treatments*

Experimental procedures followed the guidelines of the Canadian Council on Animal Care (2009) and were approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre of Sherbrooke, Québec, Canada (# 400). All animals were cared for and slaughtered according to the recommended code of practice of Agriculture Canada (1993).

Eighteen Yorkshire-Landrace gilts were selected for this study at 96.1 ± 4.6 kg BW and 135 to 170 d of age and grouped in pens (1.5 x 2.5 m per animal, half-slatted concrete flooring) of 6 to 7 animals until the first estrus was detected. For at least 14 d, they were fed a basal breeding / gestation diet ad libitum (Table 1), without Se and pyridoxine supplements but in excess of the recommended NRC (1998) requirements for all other ingredients. Estrus detection was initially performed by introducing a young boar (8 to 12 months of age) into the pen once daily (10 min), but was increased to twice daily (10 min each; between 08:00 and 09:00 h and from 16:00 to 17:00 h) for the detection of the fifth estrus. From the onset of the first estrus, gilts were placed into individual stalls (0.6 x 2.2 m, half-slatted concrete flooring), their daily feed allowance was limited to 2.8 kg, and on the basis of comparable BW and blood concentrations of Se, they were assigned randomly to one of 3 experimental diets (n = 6 per treatment): 1) basal diet (Table 1) containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine, respectively, top-dressed with 50 g of ground corn without supplemental Se or pyridoxine (CONT); 2) the basal diet top-dressed with 50 g of ground corn with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of MSe as sodium selenite, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA) (MSe$B_6$10); and 3) the basal diet top-dressed with 50 g of ground corn

with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of OSe as Se-enriched yeast, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine ($OSeB_6 10$). At the fifth estrus, all gilts were inseminated with 85 mL of semen (3×10^9 live sperm cells pooled from the same 3 Duroc boars) provided by a local AI center (CIPQ Inc., St-Lambert, QC, Canada). When estrus was detected in the morning, gilts were inseminated twice, 8 and 24 h later. When estrus was detected in the afternoon, the 2 inseminations were done 16 and 24 h later. Gilts were sacrificed 5 d after the first insemination. Average BW was 138.5 ± 6.3 kg and 181.3 ± 6.3 kg at the initiation of treatment and the end of the experiment, respectively.

Sampling

Blood samples were collected into EDTA-containing tubes (10 mL; Becton Dickinson and Co., Rutherford, NJ) by jugular vein venipuncture from all gilts at arrival to the research Centre, on the d after onset of each estrus, and at slaughter. Blood samples were stored at - 20°C for determination of Se concentrations. Blood plasma and erythrocyte samples were obtained after centrifugation of blood at 1800 x g for 12 min at 4ºC, and stored at - 20ºC and at - 80°C for determination of pyridoxal-5-phosphate (P-5-P) and SeGPX activity, respectively.

At slaughter, the reproductive tract, liver, and kidneys were collected from all gilts (6 per treatment). Liver weight and ovulation rate (number of corpora lutea (CL)) were recorded. Samples of liver and kidney tissue were collected and frozen in liquid nitrogen.

Embryo (d 5) collection and storage

Both uterine horns were immediately flushed twice with 20 ml of PBS/BSA (37ºC) and the flushing collected into 50 ml Falcon tubes and kept at 37ºC. In laboratory, flushings were transferred to a Petri dish (37ºC) and embryos were harvested using a dissection microscope. Each embryo was transferred to another Petri dish (37ºC) previously prepared with microdroplets of mDPBS/BSA (modified Dulbecco PBS: PBS solution added D-glucose, sodium pyruvate, magnesium chloride ($MgCl_2.6H_2O$), and calcium chloride ($CaCl_2.2H_2O$)). Embryos were classified according to their developmental stage as 2-4 cells, 4-8 cells, morula, compact morula, early blastocyst, blastocyst or expanded blastocyst. Groups of 3-6 similar stage embryos were transferred to a Petri dish previously prepared with mDPBS droplets without BSA under mineral oil (37ºC). Then, each group of embryos were transferred to a corresponding microcentrifuge tube with no more than 20 ul of mDPBS without BSA and frozen immediately in dry ice and kept at - 80ºC until analysis. Samples of the first flushing of each uterine horn were frozen at - 20ºC for determination of Se and P-5-P concentrations. All gilts were pregnant and available for embryo harvesting but as the microarray used was specific for PEB, litters had to be selected based on the developmental stage of embryos for suitable biomolecular analysis. Pools of 3-6 expanded blastocysts from the same gilt could be finally constituted from 11 gilts. They were distributed as follow: 4, 4, and 3 in CONT, $MSeB_6$10, and $OSeB_6$10 fed gilts, respectively.

Laboratory analysis

Measurements of Se were done using a fluorimetric method adapted by Giguère *et al.* (2005) from technique of Sheehan and Gao (1990) and P-5-P was determined using a fluorimetric method adapted by Matte *et al.* (1997) from the technique of Srivastava and Beutler (1973). SeGPX activity was determined in blood plasma using the spectrophotometric method described by Gunzler and Flohé (1985). Activities are reported as milliunits (mU) per mg of protein with 1 unit equal to 1 µmol NADPH oxidized per minute.

Total RNA extraction

The ArcturusW PicoPureW RNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA) was used for RNA extraction on each individual sample tube containing 3-6 embryos. High-quality total RNA was obtained from each sample after DNase treatment using RNase-Free DNase kit according to the protocol from Qiagen (Mississauga, On, Canada). Total RNA quality was evaluated with an Agilent 2100 Bioanalyzer using RNA 6000 Pico kit (Agilent Technologies, Mississauga, ON, Canada). Ribonucleic Acid Integrity Number (RIN) index was used as a numerical assessment of the integrity of RNA and ranged between 7.8 and 9.7. RNA amplification (RiboAmp HSPlus kit; Applied Biosystems, Carlsbad, CA, USA) was used to amplify the low quantities of total RNA isolated from the samples, with only 0.6 to 3.6 ng of total RNA available for amplification. Antisense RNA (aRNA) was generated from each sample and the quality of each amplified aRNA sample was checked using an RNA 6000 Nano kit (Agilent Technologies, Mississauga, ON, Canada). For all good quality samples, Nanodrop ND-

1000 (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the aRNA concentration.

Microarray procedure and analysis

The porcine embryo-specific microarray platform generated by Agilent (Tsoi *et al.*, 2012) was used in this study. The platform information has been previously deposited and is available from NCBI the Gene Expression Omnibus (GEO) repository with GEO ID: GPL14925.

A two-color microarray with a dye-swap replicate was performed for the $MSeB_6$10 vs CONT and $OSeB_6$10 vs CONT comparison to identify all the differentially expressed (DE) genes. Four biological replicates for the $MSeB_6$10 vs CONT and three biological and one technical replicate for the $OSeB_6$10 vs CONT comparisons were used in the experimental design. Additionally, a reference design (Konig *et al.*, 2004) was chosen using the same CONT group used in the previous comparisons as a reference for a reliable indirect comparison of gene expression for $MSeB_6$10 vs $OSeB_6$10.

All the details regarding probe labelling, hybridization and washing were previously described by (Tsoi *et al.*, 2012) and Zhou *et al.* (2014). In general, concentrations of the labelled probes and their efficiencies (DOL) were determined by Nanodrop ND-1000 with an average of 125.08 ng/µL and 1.2, respectively.

Immediately after the final washing and drying procedures, arrays were scanned at 5 µm resolution with an Axon 4200AL scanner (635 nm for Cy5 and at 532 nm for Cy3) using the autoscan feature from the default setting, and images were analysed with the Gene Pix Pro 6.0 software (Molecular Device, Sunnyvale, CA 94089 USA). The

analysed images were manually edited for any spots with hybridization artefacts and flagged for exclusion. The data from spot intensity, background subtraction, and normalization was saved in the GenePix Results (GPR) format for further data analysis.

The updated annotation with all the DE genes (adjusted P value ≤ 0.05 and log2 fold change ≥ 1.7 or ≤ -1.7), matching with the original probe ID and sequences, was performed first through NCBI Basic Local Alignment Search Tool (BLAST) with the porcine RefSeq RNA (51,160 sequences) and ESTs (1,676,424 sequences) database. Similar search was performed by using the pig accession numbers with the human RefSeq RNA (91,603 sequences) to obtain the gene symbols (GS) (Supplemental file 1) for further Gene Ontology Enrichment Analysis.

Bioinformatics tools and analysis

The Venny interactive tool to associate DE gene lists with Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/) was used to identify unique GS from each comparison. The list of unique human GS obtained from Venny analysis were further uploaded into the GORILLA Classification System (http://cbl-gorilla.cs.technion.ac.il/) to identify biological processes most related to each comparison (Eden *et al.*, 2009).

A list of 15,760 unique GS from re-annotated EMPV1 was used as background when using two unranked lists of genes (target and background lists) as a running mode during the search. Differences were considered significant at FDR q-value < 0.05.

Quantitative Real-Time RT-PCR (RT-qPCR) and analysis

A two-step RT-qPCR was performed on the same aRNA samples used for the microarray experiment. Two micrograms of aRNA was obtained from each individual sample with three biological samples from each group (CONT, n = 3; $OSeB_6$10, n = 3) to synthesise cDNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies Inc., Burlington, ON, Canada). Three technical replicates were performed for each biological sample using the KAPASYBFAST qPCR kit (Kapa Biosystems, Inc. Wilmington, MA, USA) with diluted cDNA as a template from a range of 100, 50, 25, 5, 1, and 0.25 ng. RT-qPCR conditions strictly followed the manual provided by the kit manufacturer. A reverse transcription without reverse transcriptase (negative control) was also performed together from the same plate with the ABI 7900HT PCR system. Melting curves and primer efficiencies were obtained from SDS2.3 software installed in the system by performing auto-setting for threshold cycle (Ct) and baseline calculation.

Two control genes, hypoxanthine phosphoribosyltransferase 1 (HPRT1) and peptidylprolyl isomerase A (*PPIA*), were selected according to RT² ProfilerPCR Array Pig Housekeeping Genes (Qiagen Inc. Toronto, ON, Canada). Of the two genes identified, *PPIA* was selected as the reference gene due to the lower stability measurement value (Vandesompele *et al.*, 2002) compared to *HPRT1*. Based on the microarray data, 4 DE genes related to ROS disposal systems: coenzyme Q6 monooxygenase (*COQ6*), glutaredoxin-3 (*GLRX3*), peroxiredoxin-4 (*PRDX4*) and thioredoxin (*TXN*) and 2 genes involved in B_6 (pyridoxine kinase (*PDXK*)) or Se metabolism (Sep (O-phosphoserine) tRNA:selenocysteine tRNA synthase (*SEPSECS*)) were chosen for RT-qPCR validation. The mRNA expressions of these genes were normalized with *PPIA*. The factor of up-regulation is equal to the given value in the

Randomization Data Output Box and the down-regulation factor is illustrated as a reciprocal value (Pfaffl *et al.*, 2002).

High RT-qPCR efficiencies (99.00 - 99.99%) and a single peak after melting curve analysis for all housekeeping genes and genes of interest were confirmed. The sequence information of the primers is presented on Table 2. The total expression ratio of the 6 genes of interest was tested for significance between CONT and $OSeB_6$10.

Statistical analysis

Data were analyzed using the SAS procedure for mixed models (SAS Inst. Inc., Cary, NC) (Littell *et al.*, 1996) according to a randomized arrangement of treatments in blocks, with the 3 dietary treatments as the main independent variables. The model was: $Yij = \mu + Fj + eij$, where Yij = dependent variable, Fj = dietary treatments and eij = residual error. The gilt was considered the experimental unit. The residual error term was used to test the treatment effects. For the analysis of blood Se, blood plasma SeGPX activity and blood erythrocyte P-5-P concentration, sampling times were added to the model as a second factor and were analyzed using repeated option of the MIXED procedure of SAS. Sampling times were considered during the whole experiment (6 samples, from the first estrus to slaughter). A priori comparisons were done between CONT and MSe + OSe groups (SUPPL) and also between MSe and OSe groups (Se source). Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 \leq P \leq 0.10$, and all results are expressed as adjusted means ± SEM.

Microarray data statistical analysis was performed using FlexArray (version 1.6.2 - http://genomequebec.mcgill.ca/FlexArray), as described previously by Zhou *et al.*

(2014) using a default setting of P value = 0.05 and fold-change threshold = 2.0, respectively. In general, after executing the correct background algorithm using the simple subtraction method, further normalizations within (loess) and between arrays were performed. The normalized log2 ratio of all three comparisons was submitted to NCBI GEO Database (GSE51249). Further statistical analysis using the "limma" package (Smyth, 2005), the Benjamini and Hochberg false discovery rate (BH-FDR), and the multiple comparison correction condition (Benjamini and Hochberg, 1995) along with dye effect correction was performed to identify DE genes.

For RT-qPCR analysis, the Relative Expression Software Tool 2009 (REST; http://rest.gene-quantification.info/) was used to implement a randomized test (Pfaffl *et al.*, 2002) and to assess statistical significance of the up- or down-regulation of the target genes after normalization to the reference gene. Statistical analyses were considered significant at $P \leq 0.05$.

RESULTS

Blood measurements

Whole blood Se concentrations expressed as increases relative to pre-treatment values at estrus 1 were lower in CONT than in supplemented gilts (CONT vs SUPPL, $P < 0.01$), and greater in OSe gilts than in MSe (Se source, $P < 0.05$; Figure 1). Globally, from the first estrus to 5 d of gestation, whole blood Se decreased by 2.7 % for CONT and increased by 4.9 % and 15.4 % for $MSeB_6$10 and $OSeB_6$10, respectively (treatment X time interaction, $P < 0.05$). During the complete experimental period, lower

concentrations of P-5-P in blood erythrocytes were found in CONT than in supplemented gilts (CONT vs SUPPL, $P < 0.05$; Figure 2).

SeGPX activity in blood plasma tended to increase during the experimental period (time effect, $P < 0.06$) but no treatment effect was found ($P = 0.57$). Average values were 39.36 ± 2.29, 43.84 ± 4.50, and 42.54 ± 2.33 mU/g protein at first and fifth estrus, and at slaughter, respectively.

Physiological and reproduction measurements at slaughter

There was no treatment effects (Table 3) on liver weight ($P \geq 0.17$), liver Se concentration ($P \geq 0.21$), total liver Se ($P \geq 0.24$), kidney Se concentration ($P \geq 0.24$), ovulation rate ($P \geq 0.24$), embryos recovery rate ($P \geq 0.19$), viable embryos ($P \geq 0.11$), degenerated embryos ($P \geq 0.14$), early stage embryos (from 2-4 cell to compact morula) ($P \geq 0.81$), advanced stage embryos (from early blastocyst to expanded blastocyst) ($P \geq 0.18$), and total number of embryos ($P \geq 0.25$). In the uterine flushes, P-5-P concentrations were similar among treatments ($P \geq 0.13$) but Se was not detectable (< 10 ng/mL).

Differential gene expression profile

The Venn diagram including the 3 comparisons is presented in the Figure 3. The comparisons $MSeB_6$10 vs CONT and $OSeB_6$10 vs CONT correspond to the synergy of OSe or MSe with B_6, whereas the $OSeB_6$10 vs $MSeB_6$10 comparison excludes the B_6 influence and represents a specific effect of OSe vs MSe.

The direct comparison $MSeB_6$10 vs CONT showed a total of 10 DE genes in the PEB, with 9 up-regulated (90.0%) and 1 down-regulated gene (10.0%) in $MSeB_6$10. Only 2 genes had an absolute (- or +) fold change ≥ 2. All those genes are presented in Table 4. For $OSeB_6$10 vs CONT comparison, a total of 247 genes were DE, with 185 up-regulated (75.3%) and 62 down-regulated genes (24.7%) in $OSeB_6$10. Among these genes, 119 had an absolute fold change ≥ 2, and 8 of them were ≥ 3. The 10 most up- and down-regulated genes are listed in Table 5. Regarding the reference design comparison ($OSeB_6$10 vs $MSeB_6$10), there were 96 DE genes. $OSeB_6$10 had 60 up-regulated (62.5%) and 36 down-regulated genes (37.5%), compared to $MSeB_6$10. A total of 44 genes had an absolute fold change ≥ 2, and 4 of them were ≥ 3. The 10 most up- and down-regulated genes are listed in Table 6.

For the complete list of genes related to each comparison see Supplemental file 2.

Biological process analysis of the unique DE genes

For the analysis of biological processes related to DE genes in PEB after maternal supplementations, the common unique GS between comparisons were excluded and only the exclusive unique GS of each comparison were selected (6, 173 and 25 exclusive genes for $MSeB_6$10 vs CONT, $OSeB_6$10 vs CONT, and $OSeB_6$10 vs $MSeB_6$10 comparisons, respectively) and uploaded to GORILLA.

Regarding the $OSeB_6$10 vs CONT comparison, 4 distinct biological processes (gene expression, translation, regulation of translation, and mitotic cell cycle) were stimulated (FDR q-value < 0.05). When comparing $MSeB_6$10 vs CONT and $OSeB_6$10 vs

MSeB$_6$10, no biological process was selectively stimulated by MSeB$_6$10 and OSeB$_6$10, respectively (FDR q-value > 0.05).

Expression of genes involved in glutathione (GSH) and SeGPX metabolism

Although not influenced by treatments, several genes related to the methionine cycle and transsulfuration pathway were identified (Table 7). Also, the genes responsible for the metabolism between forms of pyridoxine (dietary HCl-pyridoxine vs metabolic P-5-P) were identified: pyridoxamine 5'-phosphate oxidase (*PNPO*, fold change ranging from -0.2 to 0.1 and average P = 0.41) and *PDXK* (identified by RT-qPCR).

Other ROS disposal-related genes

Treatment effects on expression of genes related to ROS balance included up-regulation of *GLRX3* (1.7 fold change, P < 0.01), *PRDX4* (1.7 fold change, P < 0.01) and *COQ6* (1.7 fold change, P < 0.01) in the OSeB$_6$10 vs CONT comparison, and down-regulation of *TXN* (fold change ranging from -2.4 to -1.9, P < 0.01) and microsomal glutathione transferase 2 (*MGST2*; fold change ranging from -2.6 to -2.0, and P < 0.05) in the OSeB$_6$10 vs CONT and OSeB$_6$10 vs MSeB$_6$10 comparisons. Although not Se-dependent, it is noteworthy to mention that major antioxidant enzymes such as superoxide dismutases and catalase were not influenced by treatments.

Validation of microarray data by RT-qPCR

RT-qPCR confirmed the previous findings for *COQ6*, *GLRX3*, *PRDX4* and *TXN*, with expression trends similar to the microarray study. In contrast, RT-qPCR analysis

indicated no difference in relative *PDXK* expression among treatments (P = 0.38) and that *SEPSECS* was up-regulated in the $OSeB_6$10 group compared to CONT (P < 0.01) (Figure 4).

DISCUSSION

Blood Se concentrations slightly decreased from the first estrus until five days of gestation in CONT gilts fed the basal diet containing a natural level of 0.3 mg/kg of feed of Se. Such response occurred despite the fact that the present basal dietary Se level was largely higher than the basal level found in cereals grown in Se-deficient soils (Mahan et al., 2005) and also higher than the NRC (1998, 2012) recommendations of 0.15 mg/kg. The Se source effect on whole blood Se concentration of gilts is consistent with previous reports (Schrauzer, 2000; Svoboda *et al.*, 2008; Fortier *et al.*, 2012). The absorbed MSe is converted into functional selenoproteins containing selenocysteine (Sec), with slight storage in organs (Windisch, 2002). The organic source is either metabolized to reactive forms of Se or stored as SeMet during protein synthesis (Schrauzer, 2003).

The enrichment analysis indicated a marked difference of DE genes among $MSeB_6$10 vs CONT, $OSeB_6$10 vs CONT, and $OSeB_6$10 vs $MSeB_6$10 comparisons. Globally, $OSeB_6$10 supplementation to gilts affected PEB transcriptome more drastically than $MSeB_6$10, suggesting an additive effect of B_6 with OSe but not with MSe. Such responses might be related to the fact that the organic source of Se can become an integral part of many proteins, not only antioxidant enzymes, whereas MSe is largely directed towards antioxidant metabolism, as mentioned above. Moreover, the complexity

of the metabolic interaction between B_6 and OSe compared to MSe (Le Floc'h *et al.*, 2012) could also explain the higher number of genes affected.

Although it cannot be ruled out that feeding a chemical compound or yeast extracts as supplements may have conferred differences in genes expression, metabolic indicators such as circulating Se showed that the Se status was clearly altered and modulated by sources and levels of dietary Se. It appears unlikely that such major systemic changes on metabolism of sows were due to the yeast fraction of dietary organic Se. The marked DE differences (especially from OSeB610) would require such type of systemic route for metabolism because they were observed on organisms (expanded blastocysts) that are self-regulated and genetically dissimilar (half allogenic) from those (dams) receiving directly the treatments. In terms of Se transfer from dams to embryos, whatever the treatment, Se was undetectable in the uterine flushing. Therefore, although the pre-implantation embryo is nourished by the uterine fluids, it appears that this route of Se transfer is negligible for embryonic metabolism. However, before conception, the Se content of pre-ovulatory oocytes might have been influenced by the systemic effect of different blood Se concentrations, considering that the follicular fluid in which the oocyte matures is a product of the transfer of blood constituents (Fortune, 1994). In such way, more Se could have been available to embryos obtained from $OSeB_610$ treated gilts. Unfortunately, our Se determination technique does not allow measurements at the embryonic level.

Brennan *et al.* (2011), studying the effects of OSe and MSe dietary supplementation on gene expression profiles in oviduct tissue from broiler-breeder hens, also found that, even without differences in tissue Se concentration, gene expression

differed between the respective treatments. This would suggest that Se concentrations may not be always a reliable indicator of the impact of Se on physiological processes.

Although treatment-dependent DE was not observed, several genes of interest were identified in the methionine cycle and transsulfuration pathway, suggesting that PEB are capable of synthesizing SeGPX. However, surprisingly, at this stage of development neither $MSeB_6$10 nor $OSeB_6$10 were able to over-stimulate SeGPX synthesis. These observations are consistent with Fortier *et al.* (2012), who showed that the SeGPX content in 30-d porcine embryos did not differ between MSe and OSe maternal supplementation. These intriguing results raise many hypothesis regarding Sec evolutionary events, Se metabolism, and selenoproteins synthesis.

Leinfelder *et al.* (1988) and Bock *et al.* (1991) proposed that during the evolution of Sec within the genetic code, UGA was initially a sense codon for this amino acid and that Sec was used not only for synthesis of SeGPX but widely for several enzymes. Later in the evolutionary process, oxygen was introduced into the atmosphere which selected against the use of Sec as this amino acid is oxygen-labile. Consequently, the global utilization of Sec decreased during evolution and became limited to anaerobic environments only. As the expanded blastocyst develops under hypoxic and even almost anaerobic conditions (Fischer and Bavister, 1993), the possibility exists that Sec was required and used for global protein synthesis in preference to selenoproteins synthesis, as suggested by Gladyshev and Kryukov (2001), in anaerobic organisms.

The enzymatic activity of many antioxidative enzymes in embryos is much lower than in adults, and thus, embryos are particularly sensitive to oxidative damage (Parman *et al.*, 1999; Winn and Wells, 1999). The SeGPX system is a major antioxidant complex

in the organism, and is responsive to Se and B_6 status (Roy *et al.*, 2011), since B_6 catalyzes the conversion of SeMet to Sec, which is further incorporated into SeGPX and other selenoproteins. The RT-qPCR analysis revealed that *SEPSECS* (the gene that encodes a protein responsible for the last step in the synthesis of selenocysteinyl-tRNA(Sec)) was up-regulated in $OSeB_6$10 gilts. However, as this was the only SeGPX-related gene to be up-regulated, it cannot be stated that this overall metabolic pathway was in fact over-stimulated.

Additionally, Gardiner and Reed (1995) studying the recovery of GSH in mouse pre-implantation embryos, found that the blastocyst cannot convert sulfur-methionine into sulfur-cysteine via the cystathionine transsulfuration pathway. This is a relevant and critical finding for Se metabolism, because SeMet follows the sulfur-methionine biochemical pathway towards Sec (Sunde, 1984). Thus, SeMet would not produce Sec and, therefore, might direct Se towards general protein synthesis or methylation. Selenium compounds methylation enzymes cystathionine gamma-lyase (CTH) and indolethylamine N-methyltransferase (INMT), along with other general protein methylation enzymes glutamic-oxaloacetic transaminase 1 and 2 (*GOT1* and *GOT2*), glutamic-pyruvate transaminase (*GPT*), betaine-homocysteine S-methyltransferase (*BHMT*), and protein-L-isoaspartate O-methyltransferase (*PCMT1*) were not differentially expressed, whereas several genes related to general elongation factors were up-regulated and biological process related to translation, regulation of translation, and mitotic cell cycle were stimulated.

This finding reinforces the concept that, at this stage of development, Se is likely directed to the global protein synthesis in the PEB. The contribution of B_6 to this effect

appeared important because when the comparison was made between $OSeB_6$10 vs $MSeB_6$10, few genes related to general elongation factors were up-regulated and no biological process was selectively stimulated by $OSeB_6$10.

Se-glutathione peroxidase not only depends on Se and B_6 but also on the availability of GSH for its enzymatic activity (Ufer and Wang, 2011). According to Yoshida *et al.* (1993), the amount of GSH in the porcine oocyte exceeds the needs for fertilization, but during pre-implantation development, embryonic GSH continuously decreases and reaches a nadir at the blastocyst stage, whereas endogenous ROS are produced at their highest level at this time. These low GSH and high ROS levels are possibly required for normal differentiation (Parchment, 1993; Pierce *et al.*, 1991), in which ROS may act as an important regulatory system for apoptosis in the mouse blastocyst (Pierce *et al.*, 1991). According to Gardiner and Reed (1994), hydrogen peroxide in mouse blastocoel fluid causes apoptosis of pre-trophectodermal cells of the blastocyst, whereas the inner-cell-mass cells, destined to become the embryo, are protected from the toxic effects of hydrogen peroxide. The same authors suggest that this protection is performed via mechanisms that utilize GSH. This apparent control of embryo GSH production is illustrated by the present microarray and RT-qPCR data showing that *TXN* and *GLRX* (genes encoding proteins involved in recycling of GSH) were respectively down- and up-regulated in $OSeB_6$10 gilts.

Despite the lack of up-regulated genes related to the transsulfuration pathway of methionine towards the SeGPX system, $OSeB_6$10 maternal supplementation differently expressed other genes related to cell antioxidant defense such as those of the thioredoxin (*GLRX3*, *PRDX4* and *TXN*) and monooxygenase (*COQ6*) families. For the thioredoxin

family, these genes are involved with key regulators of redox signaling and consequently of the intracellular effects of ROS (Fisher *et al.*, 1999; Nordberg and Arnér, 2001; Ahsan *et al.*, 2009). They are also critical for the control of DNA damage, cell proliferation and differentiation (Laurent *et al.*, 1964; Schenk *et al.*, 1996; Saitoh *et al.*, 1998) in PEB. For the monooxygenase family, *COQ6* is required for the biosynthesis of ubiquinone, one of the most potent lipophilic antioxidants implicated in the protection of cell damage by ROS (Hyun *et al.*, 2006).

The identification of these DE genes is coherent with the previously described stimulation of mitotic cell cycle as a biological process in $OSeB_6$10 gilts. In this way, it is noteworthy to mention that, although not statistically significant, the greatest number of viable embryos was collected from these gilts and this, in absence of degenerated embryos (Table 3). Therefore, considering previous results reported by Fortier et al. (2012) in which enhanced 30-d embryos development was observed in OSe gilts without effect on embryo SeGPX activity, it can be hypothesized that these antioxidative defenses from the thioredoxin and monooxygenase families could act as supplementary mechanisms to the SeGPX system in PEB from $OSeB_6$10 supplemented sows and this may persist thereafter, for at least the first third of gestation.

In conclusion, maternal dietary OSe supplementation with pyridoxine considerably stimulated the transcriptome of porcine expanded blastocysts as compared to unsupplemented gilts. For the comparison between sources of Se with equivalent supplemental pyridoxine, there was also an important response in terms of DE genes, but no specific biological process was identified. Porcine expanded blastocysts are

potentially capable of synthesizing selenoproteins, including SeGPX; however, both Se sources with pyridoxine did not influence the SeGPX metabolic pathway at this stage.

In porcine expanded blastocysts from $OSeB_6$10 supplemented gilts, other members of the thioredoxin family, as well as ubiquinones, appear to complement the antioxidant defense and the regulation of cell proliferation. However, the mechanisms by which OSe combined with pyridoxine controls these processes remain to be explained.

DECLARATION OF INTEREST

There is no conflict of interest for all authors that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1 – Increase of blood selenium concentration (ng/ml) of gilts on each estrus, presented as LS means ± SEM.

CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, $MSeB_6 10$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_6 10$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

Figure 2 - Blood erythrocytic P-5-P concentration (uM) of gilts for each estrus, presented as LS means ± SE.

CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, $MSeB_6 10$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_6 10$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

SUPPL = average values of $MSeB_6 10$ + $OSeB_6 10$.

Figure 3 - Venn diagram summarizing the microarray analysis of $MSeB_6 10$ vs CONT, $OSeB_6 10$ vs CONT and $OSeB_6 10$ vs $MSeB_6 10$ comparisons.

Figure 4 - RT-qPCR expression trends for *COQ6*, *GLRX3*, *PRDX4*, *TXN*, *PDXK* and *SEPSECS* in porcine expanded blastocysts recovered from $OSeB_6$10 supplemented gilts, shown as relative gene expression to CONT (± SEM).

PPIA was used to normalize the mRNA expression levels.

$OSeB_6$10 = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

SUPPLEMENTAL DATA LEGENDS

Supplemental file 1 – Update annotation of the porcine embryo-specific microarray platform information.

Supplemental file 2 - Complete list of differentially expressed genes related to $MSeB_6$10 vs CONT, $OSeB_6$10 vs CONT, and $OSeB_6$10 vs $MSeB_6$10 comparisons.

Table 1. Composition of the basal diet (as-fed basis)[1,2].

Ingredients	Amount, %
Corn	52.6
Wheat shorts	20.0
Distillers dried grain with solubles.	10.0
Canola meal	9.7
Soybean hulls	4.0
Limestone	2.0
Salt	0.6
Monocalcium phosphate	0.5
L-Lysine	0.1
Choline	0.1
Feed curb[3]	0.1
Mineral and vitamin premix[3]	0.3

[1]The calculated compositions for ME, CP, lysine, Ca, and P of the basal diet were 2,702 kcal/kg, 14.0, 0.6, 1.0, and 0.6%, respectively.

[2]The basal Se and pyridoxine content of the diet were 0.3 mg/kg and 2.4 mg/kg, respectively (analytical values determined according to Giguère *et al.* (2005) and Matte *et al.* (2001), respectively).

[3]Inhibitor of mold growth

[4]Provided per kilogram of diet: Mn as manganous oxide, 40 mg; Zn as zinc oxide, 150 mg; Fe as ferrous sulfate, 140 mg; Cu as copper sulfate, 21 mg; I as calcium iodate, 2.0 mg; vitamin A, 14,580 IU; vitamin D, 1,500 IU; vitamin E, 44 IU; vitamin K, 2.6 mg; thiamine, 2.7 mg; riboflavin, 4.9 mg; niacin, 31 mg; pantothenic acid, 21 mg; folic acid, 10 mg; biotin, 400 µg; and vitamin B12, 25 µg.

Table 2. Primer sequences used for RT-qPCR amplifications of reference gene and genes encoding antioxidant enzymes in porcine expanded blastocysts.

Genes	Primer sequences † (5'→3')	GenBank Accession no.	Product size (bp)	Amplification efficiency (%)
HPRT1	(F) CAGTGACAGCACTTAGAGGTATT (R)GACAACAACTCACCGGAAATAATC	NM_001032376.2	126	99.6
PPIA	(F) GGTGACTTCACACGCCATAA (R) GACCCGTATGCTTCAGGATAAA	NM_214353.1	91	99.1
PRDX4	(F) GTGGCCAAGAGAAGAGCTATAA (R) GCATCTTGACCTGAG GAAGTAT	XM_005673496.1	94	99.8
COQ6	(F) CCAGCCCTTGGGTTCATATT (R) GACTGGTCATAGTTCCAGCTAAC	XM_001929106.3	136	99.3
GLRX3	(F) GGAAACAAACAGGAAGCCAAG (R) GTATGTCGAACGTCTCGTAGTC	XM_005671576.1	91	99.9
PDXK	(F) CAGAGCAAGAGGGACATTGAG (R) CACAAGGACGGAAACAGACA	NM_213943.1	113	99.1
SEPSEC	(F) CAAGGTATCTGGAGCTGACAAT (R) ACCCTTCACATGTCATCAAGAA	XM_003356876.2	124	99.0
TXN	(F) CTCGTAGTGGTCGATTTCTCAG	NM_214313.2	100	99.9

Table 3. Physiological and reproduction measurements at slaughter.

Measurements	CONT	MSeB610	OSeB610	SEM	A priori comparisons	
					CONT vs SUPPL	MSeB610 vs OSeB610
Uterine flush selenium concentration (ng/ml)	-	-	-	-	-	-
Uterine flush pyridoxal-5-phosphate (μM)	0.33	0.50	0.17	0.16	0.99	0.13
Liver weight (kg)	1.93	1.78	1.91	60.40	0.26	0.17
Liver selenium concentration (μg/g)	1.07	1.14	1.21	0.07	0.21	0.47
Total liver selenium concentration (mg)	2.08	2.04	2.32	0.02	0.60	0.24
Kidney selenium concentration (μg/g)	3.22	3.40	3.63	0.02	0.24	0.44
Ovulation rate/ number of corpora lutea (n)	18.83	18.83	19.67	1.16	0.86	0.24
Embryo recovery rate (%)	76.92	85.61	92.74	7.17	0.19	0.50
Viable embryos (n)	14.17	13.50	18.17	1.88	0.48	0.11
Degenerated embryos (n)	0.50	2.00	0.00	0.96	0.65	0.14
Early stage embryos (n)	3.33	4.30	3.67	2.15	0.81	0.83
Advanced stage embryos (n)	10.83	9.17	14.50	2.62	0.76	0.18
Total embryos (n)	14.67	15.50	18.17	1.60	0.28	0.25

CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).
SUPPL = average values of $MSeB_610$ + $OSeB_610$.

Table 4. Differentially expressed genes in expanded blastocysts of gilts supplemented with $MSeB_6$10 compared with CONT group.

Genes	Description	Fold Change	P value
	Up- and down-regulated genes		
PCBP2	Poly(rC) binding protein 2, transcript variant X6	2.1	1.73×10^{-2}
DALRD3	DALR anticodon binding domain containing 3	2.0	3.43×10^{-2}
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	1.9	1.58×10^{-2}
PAAF1	Proteasomal ATPase-associated factor 1	1.9	1.62×10^{-2}
EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	1.8	2.97×10^{-2}
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	1.7	2.81×10^{-2}
TUBA1A	Tubulin, alpha 1a	1.7	3.34×10^{-2}
HSD17B14	Hydroxysteroid (17-beta) dehydrogenase 14	1.7	2.49×10^{-2}
TMEM129	Transmembrane protein 129, transcript variant 1	1.7	4.76×10^{-2}
HAAO	3-hydroxyanthranilate 3,4-dioxygenase	-1.9	8.79×10^{-3}

Table 5. Top ten up- and down-regulated genes in expanded blastocysts of gilts supplemented with $OSeB_6$10 compared with CONT group.

Genes	Description	Fold Change	P value
	Up-regulated genes		
ETNPPL	Ethanolamine-phosphate phospho-lyase	3.9	2.13×10^{-5}
AK4	Adenylate kinase 3-like 1	3.1	1.62×10^{-4}
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	3.0	7.57×10^{-6}
CDV3	Protein CDV3 homolog (LOC100627162)	3.0	1.24×10^{-5}
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	2.9	1.01×10^{-4}
IL6	Interleukin 6 (interferon, beta 2)	2.9	9.14×10^{-4}
HENMT1	Small RNA 2'-O-methyltransferase-like (LOC100152743)	2.9	1.48×10^{-4}
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	2.8	2.91×10^{-6}
ST3GAL5	Lactosylceramide alpha-2,3-sialyltransferase-like	2.8	3.05×10^{-5}
ATP6V0D2	ATPase, H+ transporting, lysosomal, V0 subunit d2	2.7	1.35×10^{-5}
	Down-regulated genes		
CHAC1	ChaC, cation transport regulator homolog 1	-5.0	2.38×10^{-3}
STC2	Stanniocalcin 2	-3.6	5.31×10^{-4}
MT1L	Metallothionein-1C-like (LOC100739663)	-3.0	3.18×10^{-5}
NLN	Neurolysin (metallopeptidase M3 family)	-3.0	4.16×10^{-6}
ASNS	Asparagine synthetase (glutamine-hydrolyzing)	-2.9	3.24×10^{-6}
RGN	Regucalcin-like (LOC100523295)	-2.9	2.32×10^{-4}
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	-2.8	2.87×10^{-6}
MGST2	Microsomal glutathione S-transferase 2	-2.6	2.21×10^{-5}
AIF1	Allograft inflammatory factor 1	-2.6	1.36×10^{-3}
MGST2	Microsomal glutathione S-transferase 2	-2.5	4.26×10^{-5}

Table 6. Top ten up- and down-regulated genes in expanded blastocysts of gilts supplemented with $OSeB_6$10 compared with $MSeB_6$10 group.

Genes	Description	Fold Change	P value
	Up-regulated $OSeB_6$10 genes		
ETNPPL	Ethanolamine-phosphate phospho-lyase	3.4	2.18×10^{-3}
AK4	Adenylate kinase 3-like 1	3.0	2.01×10^{-3}
IL6	Interleukin 6 (interferon, beta 2)	2.8	2.21×10^{-3}
ATP6V0D2	ATPase, H+ transporting, lysosomal, V0 subunit d2	2.7	1.50×10^{-3}
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	2.5	4.82×10^{-2}
RUVBL2	RuvB-like 2, transcript variant X3	2.4	2.10×10^{-2}
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	2.4	1.46×10^{-2}
ST3GAL5	Lactosylceramide alpha-2,3-sialyltransferase-like	2.4	5.25×10^{-3}
KPNA3	Karyopherin alpha 3	2.3	1.13×10^{-3}
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	2.2	1.16×10^{-2}
	Down-regulated $OSeB_6$10 genes		
CHAC1	ChaC, cation transport regulator homolog 1	-4.0	6.72×10^{-3}
STC2	Stanniocalcin 2	-3.4	8.99×10^{-3}
ASNS	Asparagine synthetase (glutamine-hydrolyzing)	-2.9	8.15×10^{-3}
RGN	Regucalcin transcript variant 1	-2.8	1.74×10^{-2}
NLN	Neurolysin	-2.4	1.44×10^{-3}
SLC7A9	Solute carrier family 7, member 9	-2.4	4.36×10^{-2}
MT1L	Metallothionein 1L	-2.3	9.89×10^{-3}
IDI1	Isopentenyl-diphosphate delta isomerase 1	-2.3	1.20×10^{-3}
RGN	Regucalcin, transcript variant 2	-2.2	4.30×10^{-2}
MINOS1	Mitochondrial inner membrane organizing system 1	-2.2	1.39×10^{-3}

Table 7. Sulfur and Se antioxidation related genes expressed in the porcine expanded blastocyst.

GS	Description	A-value[a]	SD
	Common metabolism for Se and sulfur		
MAT1A	S-adenosylmethionine synthetase isoform type-1	6.1	0.3
MAT2A	S-adenosylmethionine synthetase isoform type-2	9.1	1.8
AHCY	S-adenosylhomocysteine hydrolase	9.5	1.0
AHCYL1	S-adenosylhomocysteine hydrolase-like 1	6.3	0.5
AHCYL2	S-adenosylhomocysteine hydrolase-like 2	6.4	0.4
CTH	cystathionine gamma-lyase	7.7	2.9
	GSH synthesis, recycling, and utilization		
GCLC	glutamate--cysteine ligase catalytic subunit	8.0	3.8
GCLM	gamma-glutamylcysteine synthetase regulatory subunit	8.9	1.7
GSS	glutathione synthase	6.9	0.6
GSR	glutathione reductase	7.0	0.8
GGT1	gamma-glutamyltransferase 1	6.5	1.0
GGT5	gamma-glutamyltransferase 5	6.5	0.6
GGT6	gamma-glutamyltransferase 6	6.2	0.4
GGT7	gamma-glutamyltransferase 7	6.2	0.3
GPX5	glutathione peroxidase 5	6.2	0.3
GPX8	glutathione peroxidase 8	6.2	0.5
GSTA1	glutathione S-transferase alpha 1	6.1	0.4
GSTA4	glutathione S-transferase alpha 4	7.3	0.9
GSTA5	glutathione S-transferase alpha 5	6.7	0.5
GSTCD	Glutathione S-transferase, C-terminal containing domain	7.1	2.2
GSTK1	glutathione S-transferase kappa 1	7.1	0.5
GSTM1	glutathione S-transferase mu 1	6.6	0.8
GSTM3	glutathione S-transferase mu 3	7.7	1.7
GSTO1	glutathione S-transferase omega 1	9.4	1.8
GSTO2	glutathione S-transferase omega 2	7.6	0.8
GSTP1	glutathione S-transferase pi 1	9.8	2.4
GSTT1	glutathione S-transferase theta 1	6.2	1.0
GSTT4	glutathione S-transferase theta 4	6.0	0.3
GSTZ1	glutathione transferase zeta 1	6.3	0.3
MGST2	Microsomal glutathione transferase 2	8.4	2.9

MGST3	Microsomal glutathione transferase 3	7.7	0.7
PTGES	prostaglandin E synthase	9.5	2.4
PTGES2	prostaglandin E synthase 2	6.7	0.8
PTGES3	prostaglandin E synthase 3	9.8	2.9
	Exclusive metabolism of SeGPX		
SCLY	selenocysteine lyase	6.0	0.4
SEPHS2	selenophosphate synthetase 2	6.4	0.4
SARS	seryl tRNA ligase	6.9	0.9
SARS2	seryl tRNA ligase 2	6.1	0.5
PSTK	phosphoseryl-tRNA kinase	6.7	0.3
SEPSECS	Sep (O-phosphoserine) tRNA:Sec tRNA synthase	6.2	0.3
EEFSEC	eukaryotic elongation factor. selenocysteine-tRNA-specific	6.2	0.2
SECISBP2	selenocysteine insertion sequence-binding protein 2	6.6	0.6
SECISBP2L	selenocysteine insertion sequence-binding protein 2-like	6.3	0.5
	Selenoproteins		
SEPN1	selenoprotein N, variant 1	6.1	0.2
SEPP1	selenoprotein P, variant 1	6.1	0.4
DIO3	Iodothyronine 3	6.4	1.3
SELK	selenoprotein K	8.8	2.2
SELM	selenoprotein M	7.1	1.8
SELO	selenoprotein O	8.1	2.5
SELS	VCP-interacting membrane protein, variant 2	8.8	1.9
SELT	selenoprotein T	6.4	0.4
SELV	selenoprotein V	6.5	0.2
TXNRD1	thioredoxin reductase 1	6.6	0.6
TXNRD2	thioredoxin reductase 2	6.1	0.2
TXNRD3	thioredoxin reductase 3	7.0	0.9
GPX2	glutathione peroxidase 2	6.2	0.3
GPX3	glutathione peroxidase 3	7.0	1.2
GPX6	glutathione peroxidase 6	7.4	1.7

[a] The level of gene expression was indicated by the average A-value reflecting spot intensity higher than the background intensity. Average A-value from dark-corner spot intensity was consider to be background (mean A-value = 6.0 and SD = 0.3).

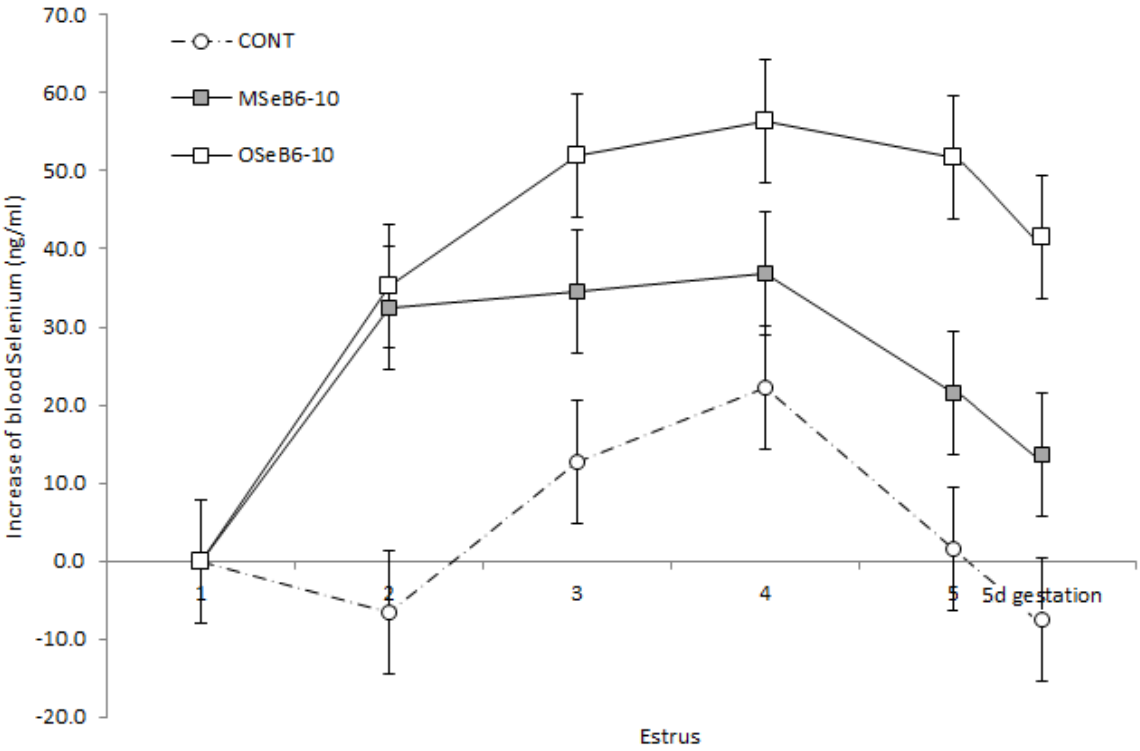
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
CONT
MSeB6-10
OSeB6-10
Increase of bloodSelenium (ng/ml)
1
2
3
4
5
5d gestation
Estrus

Figure 1

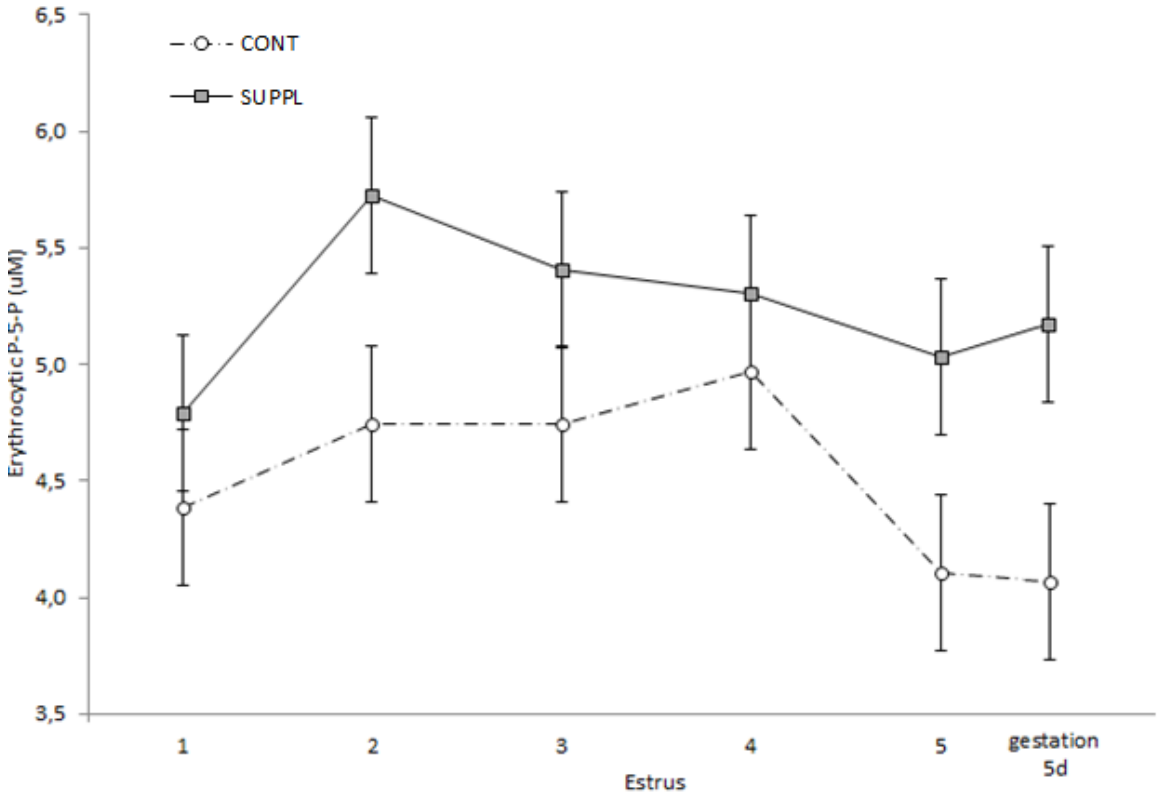
6,5
6,0
5,5
5,0
4,5
4,0
3,5
CONT
SUPPL
Erythrocytic P-5-P (uM)
1
2
3
4
5
gestation 5d
Estrus

Figure 2

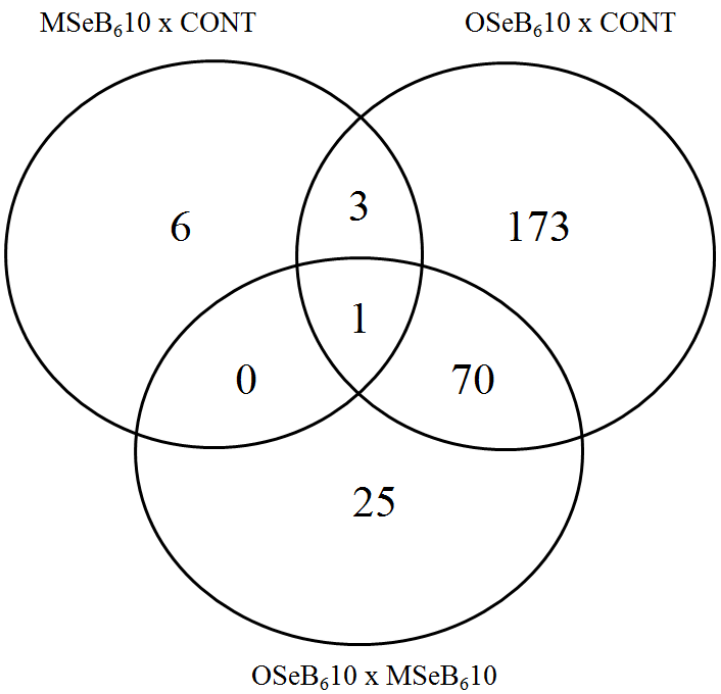
MSeB6 10 x CONT
OSeB6 10 x CONT
6
3
173
1
0
70
25
OSeB6 10 x MSeB6 10

Figure 3

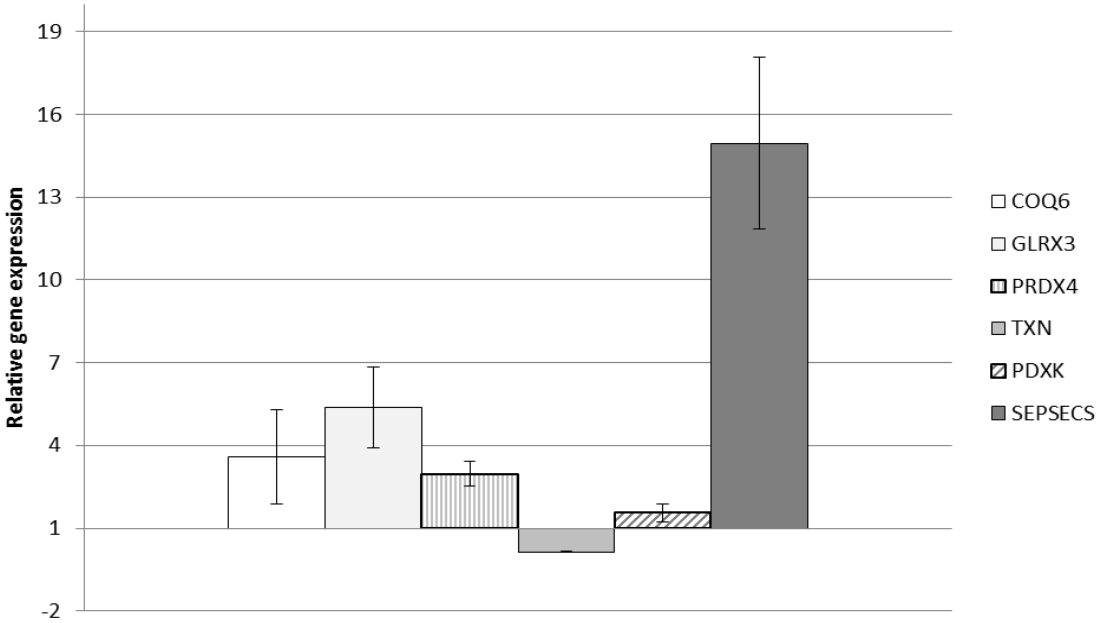
19
16
13
10
7
4
1
-2
Relative gene expression
COQ6
GLRX3
PRDX4
TXN
PDXK
SEPSECS

Figure 4

The importance of pyridoxine for the impact of the dietary selenium sources on the GPX system, embryo development, and reproductive performance in gilts[1]

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ABSTRACT: This study aimed to determine the effects of pyridoxine combined with dietary selenium (Se) on the Se-glutathione peroxidase (GPX) system, embryo development, and reproductive performance in gilts. Eighty-four gilts were randomly assigned to one of the 5 experimental diets: 1) basal diet (0.3 and 2.4 mg/kg of natural Se and pyridoxine, respectively) (CONT, n = 16); 2) basal diet + 0.3 mg/kg of Na-selenite ($MSeB_60$, n = 17); 3) diet 2 + 10 mg/kg of HCl-pyridoxine ($MSeB_610$, n = 18); 4) basal diet + 0.3 mg/kg of Se-enriched yeast ($OSeB_60$, n = 17); and 5) diet 4 + 10 mg/kg of HCl-pyridoxine ($OSeB_610$, n = 16). Blood samples were collected on the d after each estrus and at slaughter (long-term profiles), and daily from d -4 to d +3 of the fourth estrus (peri-estrus profiles). At slaughter (d 30 after the fifth estrus), liver, kidney, embryos, and corpora lutea (CL) were collected and ovulation rate assessed. For long-term profiles, CONT had lower blood Se than Se-supplemented gilts ($P < 0.01$) and OSe were higher than MSe gilts ($P < 0.01$). Lower erythrocyte pyridoxal-5-phosphate (P-5-P) concentration ($P < 0.01$) and higher plasma GPX activity ($P < 0.05$) were found in B_60 than B_610. For peri-estrus profiles, treatment effects on blood Se were similar to those for long-term profiles. Plasma P-5-P was higher in B_60 than B_610 ($P < 0.01$), with $MSeB_610$ presenting the highest concentration ($P < 0.01$). No treatment effect was observed on plasma GPX activity ($P = 0.27$). In liver and CL, CONT had lower Se concentration than Se-supplemented gilts (Se level, $P < 0.01$). In embryos, CONT had lower Se concentration than Se-supplemented gilts (Se level, $P < 0.01$) and OSe were higher than MSe gilts ($P < 0.05$). Lower litter Se content variation and weight variations were found in B_60 than B_610 ($P < 0.05$). In conclusion, B_6 level affected the GPX activity at long-term basis. The supplementation with 0.3 mg/kg of Se for gilts appears to be adequate to alleviate the oxidative stress induced by the peri-estrus period. Neither Se sources have not improved embryo quality whereas B_6 has increased litter heterogeneity.

Key words: embryo, gestation, gilt, glutathione peroxidase, pyridoxine, selenium

INTRODUCTION

The peri-estrus period is characterized by an intensified ovarian metabolism in response to hormonal regulations, which generates reactive oxygen species (**ROS**)

(Agarwal et al., 2005). Although essential for ovulation, uncontrolled oxidative stress has been linked to ovulatory dysfunction (Al-Gubory et al., 2010). Moreover, during early gestation, embryos develop under hypoxic conditions, but the rise in O_2 tension with placentation is associated with higher expression of ROS markers (Jauniaux et al., 2000). These oxidative conditions have been related with inhibition of oocytes (Guerin et al., 2001) and embryos development (Ufer and Wang, 2011). Indeed, the peri-placentation period in pigs is associated with increased loss of embryo-attachment sites (Gonzalez-Añover et al., 2011). Therefore, these detrimental metabolic by-products must be neutralized locally by antioxidants such as the glutathione peroxidase (**GPX**) system.

Recently, Fortier et al. (2012) and Dalto et al. (2014b) have shown the effects of dietary selenium (**Se**) on the metabolic control of GPX in pigs during the peri-estrus period, in which blood GPX activity decreased during the peri-estrus period in CONT gilts, whereas it was maintained and enhanced by dietary inorganic (**MSe**) and organic Se (**OSe**) supplementation, respectively. Such regulation was more apparent in OSe gilts, suggesting a shift of this enzyme in response to the oxidative pressure of ovulation. This response might be related to the dependency of OSe on vitamin B_6 to direct Se to GPX through the transsulfuration pathway, whereas MSe short-circuits this process and the regulation of GPX by redox changes (Yasumoto et al., 1979; Johansson et al., 2005).

The present experiment aimed to determine the importance of vitamin B_6 and Se metabolisms interactions for an adequate flow of Se towards the GPX system during the peri-estrus period, and its impact on maternal Se transfer to embryos, embryonic GPX status, and litter development parameters in early gestation of gilts.

MATERIALS AND METHODS

Experimental procedures followed the guidelines of the Canadian Council on Animal Care (2009) and were approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre of Sherbrooke, Québec, Canada (# 400). All animals were cared for and slaughtered according to the recommended code of practice of Agriculture Canada (2014).

Animals and treatments

Eight-four Yorshire-Landrace gilts were selected for this study at 91.4 ± 1.0 kg BW and 135 to 170 days of age. They were grouped in pens (1.5 x 2.5 m, half-slatted concrete flooring) of 6 to 7 animals until the first estrus was detected. For at least 14 d, they were fed *ad libitum* a basal breeding / gestation diet (Table 1) without Se and pyridoxine supplements but in excess of the recommended NRC (1998) levels for all other nutrients. From the first estrus the daily feed allowance was controlled and limited to 2.8 kg. Estrus detection was performed by introducing a young boar (8 to 12 months of age) into the pen once daily for the first 4 estruses (10 min; between 08:00 and 09:00 h). From the first estrus, gilts were placed into individual stalls (0.6 x 2.2 m, half-slatted concrete flooring) and were randomly assigned (according to their BW and blood concentration of Se) to one of the 5 experimental diets: 1) basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively (Table 1), top-dressed with 50 g of ground corn without supplemental Se or pyridoxine (**CONT**, n = 16); 2) basal diet top-dressed with 50 g of ground corn with supplemental Se, providing an equivalent of 0.3 mg/kg of feed of MSe as sodium selenite (214485 Sigma-Aldrich, St-Louis, MO, USA), and without pyridoxine (**MSeB$_6$0**, n = 17); 3) basal diet top-dressed with 50 g of ground corn with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of MSe as sodium selenite, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA) (**MSeB$_6$10**, n = 18); 4) basal diet top-dressed with 50 g of ground corn with supplemental Se, providing an equivalent of 0.3 mg/kg of feed of OSe as Se-enriched yeast (Alltech, Lexington, KY,USA), and without pyridoxine (**OSeB$_6$0**, n = 17); and 5) basal diet top-dressed with 50 g of ground corn with supplemental Se and pyridoxine, providing an equivalent of 0.3 mg/kg of feed of OSe as Se-enriched yeast, and 10 mg/kg of feed of pyridoxine, as hydro-chloride pyridoxine (**OSeB$_6$10**, n = 16). The fifth estrus was detected twice a day by introducing a boar into the pen (10 min each; between 08:00 and 09:00 h and between 16:00 and 17:00 h). When estrus was detected in the morning, gilts were inseminated 8 and 24 h later. If estrus was detected in the afternoon, they were inseminated 16 and 24 h later. The inseminations were performed using 85 mL of semen (3×10^9 live sperm cells from

pooled semen of 3 Duroc boars) provided by a local AI center (CIPQ Inc., St-Lambert, Quebec, Canada). Gilts were sacrificed 30 days after the first insemination. Average BW was 116.2 ± 1.8 kg and 184.3 ± 1.3 kg at the beginning of treatment and the end of the experiment, respectively.

Sampling

Blood samples were collected into EDTA-containing tubes (10 mL; Becton Dickinson and Co., Rutherford, NJ) from the jugular vein by venipuncture on all gilts at arrival to the research Centre, on the d after each onset of estrus, and at slaughter (long-term profiles). On d 14 after the fourth estrus, 5 gilts from treatments CONT, $MSeB_60$ and $MSeB_610$, and 6 gilts from treatments $OSeB_60$ and $OSeB_610$ were cannulated at the jugular vein by a nonsurgical technique described by Matte (1999). Blood samples were then collected daily from d -4 to d +3 of the fourth onset of estrus (peri-estrus profiles). At slaughter, the reproductive tract, liver and kidneys were collected from all 77 gestating gilts (15 gilts for CONT, $MSeB_60$ and $MSeB_{61}0$, and 16 gilts for $OSeB_60$ and $OSeB_610$). Uterine horns were dissected from the mesometrium and ovaries. Liver, uterus (before and after embryo harvesting), and ovary weights, and uterine horns length were recorded. Corpora lutea (**CL**) were dissected from the ovaries and embryos harvested from the uterus. Ovulation rates (number of CL) and embryo survival (percentage of CL represented by viable embryos) were recorded. Embryos and CL were weighed and measured (diameter of CL and length of embryo). Samples of liver, kidney, and right horn endometrial tissue, as well as, all embryos individually and the CL polled by gilt were immediately frozen in liquid nitrogen and further stored at - 80°C.

Blood samples were stored at - 20°C for determination of Se concentrations. After centrifugation of blood at 1800 x g for 12 min at 4ºC, plasma was separated into aliquots and stored at - 80°C for GPX activity and at - 20°C for luteinizing hormone (**LH**), pyridoxal-5-phosphate (**P-5-P**) (peri-estrus profile), and protein analyses. Erythrocytes were stored at - 20°C for P-5-P analyses (log-term profile).

Concentration of Se was measured in samples of liver, kidney, CL, and embryos. The activity of GPX and concentrations of DNA and protein were measured in embryos. At the time of the analysis, individual embryos and polled CL were thawed and

homogenized in 2.5 and 4.0 mL of deionized water, respectively, and frozen at - 80°C (for a maximum of 2 d) for GPX activity and at - 20°C for Se, DNA, and protein measurements.

Laboratory analysis

Measurements of Se were performed using a fluorimetric method adapted by Giguère et al. (2005) from the technique of Sheehan and Gao (1990). The P-5-P concentration was determined using a fluorimetric method adapted by Matte et al. (1997) from the technique of Srivastava and Beutler (1973). The GPX activity was determined using a spectrophotometric method described by Gunzler and Flohé (1985). Activities are reported as milliunit (**mU**) per mg of protein, with 1 unit equal to 1 µmol of NADPH oxidized per minute. Quantifications of DNA and proteins were performed using the methods of Labarca and Paigen (1980) and Bradford (1976) (Bio- Rad DC Protein Assay, Mississauga, Canada), respectively.

Plasma LH and E_2 were measured using the method of Kingsbury and Rawlings (1993) as described by Fortier et al. (2012).

Statistical analysis

Data were analyzed using the SAS procedure for mixed models (SAS Inst. Inc., Cary, NC; Littell et al., 1996) according to a randomized arrangement of treatments in blocks with the 5 dietary treatments in the experimental period as the main independent variables. The model was: $Y_{ijk} = \mu + J_i + W_j + (JW)_{ij} + e_{ijk}$, where Y_{ijk} = dependent variable, μ = general mean, J_i = Se treatments, Wj = B_6 treatments, $(JW)_{ij}$ = interaction Se X B_6, and e_{ijk} = residual error. The gilt was considered the experimental unit. The residual error term was used to test the treatment effects. For the analyses of hormones and other components, sampling times were added to the model as a second factor and were analyzed using repeated option of the MIXED procedure of SAS. Sampling times were considered during the whole experiment (6 samples for long-term profiles) or during the peri-ovulatory period of the fourth estrus (8 samples for the peri-estrus profiles from d -4 to d +3; d 0 corresponds to the physiological estrus as identified by the LH peak). When treatment effects or treatment X time interaction were significant, priori comparisons

were done according to the factorial arrangement (Se source, B_6 level, and Se source X B_6 level interaction) with an additional comparison of all the 4 treatments versus CONT (Se level). Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 \leq P \leq 0.10$, and all results are expressed as adjusted means ± SEM.

RESULTS AND DISCUSSION

Long-term effects of the dietary treatments

Different blood sampling procedures, such as jugular vein catheter and vacuum puncture after snare restraint, provide different results for Se and pyridoxine concentrations, as described by (Dalto et al., 2014b). This might likely interfere with the interpretation of the present sample values collected for long-term (venipuncture) and peri-estrus (jugular vein catheter) profiles. As the present results were repeated measurements according to estrus, data from the first, second, three, and fifth estrus, and slaughter were selected for the evaluation of long-term effects whereas those from the fourth estrus were used to evaluate the peri-estrus effects.

Blood Se concentrations expressed as increases relative to pre-treatment values at estrus 1 were lower in CONT than in Se-supplemented gilts (Se level, $P < 0.01$) and greater in OSe than in MSe gilts (Se source, $P < 0.01$). Initial values were 271.4, 273.4, and 275.8 mg/ml for CONT, MSe, and OSe groups, respectively. From the first to the fifth estrus, blood Se concentration decreased 0.28 mg/ml (0.1 %) for CONT and increased 26.97 (9.8 %) and 47.18 mg/ml (17.1 %) for MSe and OSe gilts, respectively. However, from the fifth estrus until 30 d of gestation, blood Se concentration decreased 13.68 (5.1 %), 5.90 (1.2 %), and 0.54 mg/ml (0.2 %) for CONT, MSe, and OSe gilts, respectively (treatment X time interaction, $P < 0.01$; Figure 1). These effects of Se source on blood Se concentration of gilts are consistent with other authors (Svoboda et al., 2008; Fortier et al., 2012, Dalto et al., 2014b). The inorganic source of Se is directly incorporated into selenoproteins with limited tissue accumulation, whereas OSe is not only incorporated into selenoproteins (Windisch, 2002) but also deposited into body proteins following the methionine metabolism (Schrauzer, 2003).

Blood Se concentrations in CONT gilts was maintained at similar levels until the fifth estrus by the basal diet containing a natural level of 0.3 mg/kg of feed of Se, which is largely higher than the NRC (1998, 2012) recommendations of 0.15 mg/kg. However, during the first 30 d of gestation, the decrease in blood Se concentration in all treatments suggests that they were developing a negative Se balance. Other authors have found a decline of approximately 15.0 % in Se concentration of CONT gilts from gestation to parturition (Mahan and Peters, 2004), suggesting a greater demand for Se to produce selenoproteins or for Se transfer to fetal or mammary tissue during late pregnancy. However, it is noteworthy to mention that in late gestation (mainly after the 11^{th} week) blood and serum volumes increase by approximatively 25% in sows, leading to a dilution effect in all metabolites present in these samples (Matte and Girard, 1996). Hostetler and Kincaid (2004) have shown that porcine embryo Se content increases until 24 d of gestation with further decrease to a constant level, suggesting a higher Se requirement due to the oxidative conditions of the placentation period. The present results are in line with these authors and, considering that the effect of Se on blood Se levels relies on the duration of supplementation (Quesnel et al., 2008), a constant dietary Se supplementation prior to gestation appears to be essential. No effects of vitamin B_6 supplementation was observed on blood Se concentration (B_6 level, $P = 0.44$).

For long-term profiles, erythrocyte P-5-P was chosen as an indicator of vitamin B_6 status. In pigs, P-5-P is over six times more concentrated in erythrocytes than in plasma, with a saturable storage in these cells (Matte et al., 2001). For the whole experimental period, lower erythrocyte P-5-P concentration was found in B_60 than B_610 gilts (B_6 level, $P < 0.01$). From the first to the fifth estrus, erythrocyte P-5-P concentration increased by 3.3 % and 28.4 % for B_60 and B_610 gilts, respectively; however, from the fifth estrus until 30 d of gestation concentrations decreased by 2.2 % for B_60 and increased by 3.5 % for B_610 gilts (treatment X time interaction, $P < 0.01$). Average pre-treatment values at estrus 1 were 3.97 ± 0.16 and 4.01 ± 0.16 uM for B_60 and B_610 fed gilts, respectively. Average fifth estrus values were 4.10 ± 0.17 and 5.15 ± 0.16 uM and slaughter values were 4.01 ± 0.17 and 5.33 ± 0.18 uM for B_60 and B_610 fed gilts, respectively. The dietary vitamin B_6 concentration in the basal diet (2.4 mg/kg; analytical value expressed as pyridoxine equivalent, i.e. sum of pyridoxamine, pyridoxal,

and pyridoxine in the mix feed) has supplied the gilts requirements until the fifth estrus, as shown by erythrocyte B_6 storage. However, the observed decrease in erythrocyte P-5-P deposition during early gestation suggests a higher B_6 mobilization in this period. This gestational utilization of B_6 appears to be responsive to the B_6 status of the animal, in which the B_6 deposition during gestation was more marked reduced in $B_6$10 (28.4 % and before insemination and 3.5 % during gestation) than in $B_6$0 gilts (3.3 % before insemination and -2.2 % during gestation). There was no overall effect of Se supplementation on erythrocyte P-5-P concentration (Se source, $P = 0.72$).

No treatment effect was observed on plasma protein concentration ($P = 0.70$). However, a time effect was found ($P < 0.01$) in which concentrations remained initially stable in all treatments until the fifth estrus (79.15 ± 1.36 and 79.34 ± 1.37 mg/ml for the first and fifth estrus, respectively), decreasing thereafter on d 30 of gestation (74.93 ± 1.40 mg/ml). Although fetal demand for nutrients occurs primarily during the last half of gestation, adjustments in nutrient metabolism are apparent within the first weeks of pregnancy (Hytten and Chamberlain, 1980). Considering that the blood volume increases mainly in late gestation (Matte and Girard, 1996) and that albumin is a carrier protein for many nutrients (King, 2000), it is likely that this decreased plasma protein concentration is related to the natural drop in albumin concentration observed early in gestation.

No effects of Se level and/or source were found on plasma GPX activity ($P = 0.17$). However, $B_6$0 had higher plasma GPX activity than $B_6$10 gilts (B_6 level, $P < 0.01$; Figure 2). In the literature, responses of GPX activity to Se supplementation in pigs are rather inconsistent (Kim and Mahan, 2001; Yoon and McMillan, 2006; Svoboda et al., 2008; Fortier et al., 2012; Dalto et al., 2014b). According to Zhan et al. (2010), this might be due to the wide variation of the premix Se-enriched yeast content in the different studies. However, the physiological status of the animals (post-weanling piglets, grower-finisher pigs, and gestation gilts), and consequent different metabolic oxidative pressure, might influence the response of this antioxidant enzyme as well. Mahan et al. (1999) suggested that the GPX activity in Se-deficient animals responds faster to MSe than to OSe supplementation, but in continuously supplemented animals, both sources have similar results. In the present study, all animals were fed a basal diet containing 0.3 mg/kg of natural Se for at least 14 d before the initiation of treatments supplementation.

Therefore, it suggests that 0.3 mg/kg of Se was sufficient to support the requirements for GPX activity at long-term basis. Some authors have shown the effects of B_6 supplementation on plasma and erythrocyte GPX activity in rats (Yasumoto et al., 1979; Yin et al., 1991) and whole blood in pigs (Dalto et al., 2014b); however, this is the first study presenting the effects of B_6 on plasma GPX activity of pigs. According to Yin et al. (1992), B_6-deficient rats had higher GPX activity in plasma, whereas B_6-supplemented animals (2.5 mg/kg of diet of pyridoxine-HCl) had higher erythrocyte GPX activity, with no differences between MSe and OSe. Therefore, in the present study, it is likely that the lower plasma GPX activity in $B_6$10 gilts was due to a directed GPX synthesis to the erythrocytes, leading to basal GPX activity in plasma, and not to a higher plasma GPX activity in $B_6$0 gilts.

Effects of the dietary treatments during the peri-estrus period

During the peri-estrus period, CONT had lower blood Se concentration than Se-supplemented gilts (Se level, $P < 0.01$) and OSe were greater than MSe gilts (Se source, $P < 0.01$; Figure 3). Although no treatment X time interaction was found ($P = 0.86$), from d -4 to d -1 prior to ovulation, blood Se concentration decreased in all treatments (2.9 %); however, from d -1 to d +3 Se concentration increased by 2.2 %. Overall, Se concentrations decreased by 0.76 % from d -4 to d +3 (time effect, $P < 0.05$). This result further supports the increased utilization of Se during the peri-estrus period in gilts as proposed by Fortier et al. (2012). Dalto et al. (2014b) presented similar results in which blood Se concentrations dropped in all treatments until d -1 before ovulation, increasing thereafter but only in Se-supplemented gilts. In the present study, Se concentrations increased after ovulation not only Se-supplemented but also CONT gilts. This is in line with the long-term effects in which the basal dietary provision of Se was adequate to cover the metabolic needs for this mineral. There was no overall effect of B_6 supplementation on blood Se concentration (B_6 level, $P = 0.18$).

Conversely to the use of erythrocytes for long-term profiles, plasma P-5-P was chosen for the peri-estrus profile because it readily responds to short-term metabolic mobilization and/or utilization of P-5-P (Bender, 2003). During the peri-estrus period,

lower plasma P-5-P concentration was found in $B_6$0 than $B_6$10 gilts (B_6 level, $P < 0.01$; Figure 4). There was no Se source effect on plasma P-5-P concentration ($P = 0.16$); however, values were higher for MSe$B_6$10 gilts than the others (Se source X B_6 level interaction, $P < 0.01$). Although the metabolism of both Se sources relies upon B_6 catalyzed reactions, the differences between them indicate a higher utilization of B_6 by OSe. Therefore, the greater concentration of P-5-P in MSe$B_6$10 gilts might not be due to higher plasmatic accumulation in MSe$B_6$10 gilts, but to major plasmatic withdraw of B_6 in OSe$B_6$10 gilts.

Plasma protein concentration was affected by ovulation (time effect; $P < 0.01$). In all treatments, concentrations increased from d -4 until d +1 with further decrease until d +3. During this period, the breakdown of follicle walls and the release of mature ova induce an inflammatory reaction with massive recruitment of inflammatory cells and greater flow of plasma proteins to the ovulation site (Richards et al., 2002).

No treatment effect was observed on plasma GPX activity ($P = 0.27$) during the peri-estrus period, with average values of 36.21 ± 2.34 and 34.57 ± 2.41 mU/mg protein for d -4 and d +3, respectively. According to Mahan and Peters (2004) and Yoon and McMillan (2006), both sources of Se are suitable for GPX synthesis. The present results are not in line with Fortier et al. (2012) and Dalto et al. (2014b) that observed a constant decrease in blood GPX activity during the peri-estrus period in CONT gilts, suggesting a metabolic response to the oxidative burst occurring around the time of ovulation. Additionally, both authors showed that the shift of this enzyme in response to oxidative pressure of ovulation was more apparent in OSe gilts. However, different of the present study, in Fortier et al. (2012) the basal diet presented lower levels of Se (0.2 mg/kg of diet) and all gilts were fed similar levels of B_6 (3.0 mg/kg of diet), whereas in Dalto et al. (2014b) the basal diet presented lower levels of Se and pyridoxine (0.2 and 1.7 mg/kg, respectively). As commented above, despite the transient decrease in Se concentration prior to ovulation, the Se status of the gilts in all treatments was adequate. Therefore, it is possible that the present high Se level of the basal diet was sufficient to support GPX activity during the peri-estrus period. Dalto et al. (2014b), supplementing gestating gilts with 10.0 mg of B_6 /kg of diet, did not find any B_6 effect or interaction with Se treatments on blood GPX activity, suggesting that the activity of this enzyme in the blood of OSe-

treated gilts was not dependent upon the B_6 status of the animals. In contrast, Yasumoto et al. (1979) had previous shown that the positive effects of OSe on the GPX system are mediated by vitamin B_6 in vitamin B_6-deficient rats after supplementation with vitamin B_6. In the present study, differently of the long-term profiles, at the peri-estrus period B_6 supplementation did not interfere with the peri-estrus plasma GPX activity. This likely suggests a shift of this enzyme in response to the oxidative pressure of ovulation, in which B_6 no more preferably directs the GPX activity to erythrocytes relatively to plasma, as indicated by the long-term effects.

Effects of dietary treatments on organs and embryos Se content and embryos GPX activity at slaughter

There were no treatment effects on litter total Se content ($P = 0.13$), average embryo GPX activity ($P = 0.80$), and litter GPX activity variation ($P = 0.17$). Liver and CL concentrations of Se were lower in CONT than in Se-supplemented gilts (Se level, $P \leq 0.01$), but not for Se concentration in kidney ($P = 0.26$) (Table 2). According to Dalto et al. (2014b), these different responses for tissue Se level effect might be related to tissue-specific Se metabolisms. In fact, Suzuki and Ogra (2002) showed that proliferative tissues, such as liver and CL, deposit Se more efficiently than non-proliferative tissues, such as kidney. The absence of Se source effect is not in line with previous reports in which OSe supplementation has provided higher tissue deposition of this trace element (Mahan et al., 2005; Mateo et al., 2005). Although the animals used in the present study were gilts, at slaughter they were 285 to 320 days of age and were weighting an average of 184.3 ± 1.3 kg. It implies that, even with an incomplete physiological development, their tissues were more mature than of grower-finisher pigs. Mahan (2000) hypothesized that when OSe is fed to sows, less tissue Se retention would be expected because the protein turnover is slower than in immature fast growing animals. Consequently, more absorbed OSe would be available to embryos and related tissues during gestation.

The maternal Se transfer to embryos was affected by the dietary treatments. Embryos Se concentration was lower in CONT than in Se-supplemented gilts (Se level, $P < 0.01$) and greater in OSe than MSe gilts (Se source, $P < 0.05$). Previous studies have

demonstrated that maternal Se intake during gestation affects the Se concentration of neonatal tissues in pigs (Yoon and McMillan, 2006; Svoboda et al., 2008). In sheep and goats, Ghany-Hefnawy et al. (2007) have shown that the fetus obtains its Se requirements regardless of the maternal status; however, these authors did not specify the source nor the level of Se fed to the animals. The Se source effect is in line with Fortier et al. (2012); however, these authors did not find a Se level effect, suggesting that the transfer of Se to fetuses would occur later in gestation for MSe comparing to OSe. It is noteworthy to mention that marked higher levels of Se in embryos were observed in the present study comparing to Fortier et al. (2012) (1.9, 2.4, and 1.9 times higher for CONT, MSe, and OSe, respectively). Inorganic Se transport across the placental membrane has been described as an anion exchange pathway shared with sulphate (Shennan, 1988) and might influenced by factors such as the binding of other metabolites to blood proteins in both embryo and dam (Watkins, 1983). In contrast, selenoamino acids are actively transported from dam to fetus indiscriminate as methionine (Jacobssons and Oksanenh, 1966). Therefore, these results confirm that Se is transferred to porcine embryos early in gestation and strongly suggest that, in gilts, maternal MSe transfer to embryos is dependent on the maternal Se status.

Effects of dietary treatments on reproductive performance and embryos development

There were no treatment effects on the number of CL and viable embryos ($P \geq 0.34$), embryo survival ($P = 0.49$), average and total CL weight ($P \geq 0.62$), litter and average embryo weight ($P \geq 0.50$), average CL diameter ($P = 0.99$) and embryo length ($P = 0.61$), average embryo protein and DNA content ($P \geq 0.27$), litter total protein and DNA content ($P \geq 0.12$), and average embryo protein : DNA ratio ($P = 0.46$) (Table 3).

These results are not in line Dalto et al. (2014b) in which the number of CL have slightly tended to be greater in OSe$B_6$10 gilts. Although the amount of supplemental B_6 was similar to the present study (10.0 mg/kg of diet), the Se concentration of the basal diet (2.0 mg/kg of diet) was lower. According to Pappas et al. (2008), Se stimulates cells proliferation of small follicles and improves their response to the stimulatory effects of gonadotropins. In fact, in Dalto et al. (2014b) the average number of CL for CONT gilts

was 17.4 whereas in the present study this value was 19.4. Therefore, it is possible that the high Se level in the CONT diet in the present study was sufficient to induce optimal ovulation quantitatively. The absence of Se effect contrasts with Fortier et al. (2012) which found that embryos average length and weight, protein and DNA content, and total Se in the litter were or tended to be greater in OSe than MSe gilts. However, different of the present study, these authors have performed a hyperovulatory protocol, which leads to an increased oxidative pressure and possibly more marked physiological responses to antioxidant nutrients such as Se.

The individual embryo measurements allowed the calculation of within-litter variations as indicators of litter heterogeneity for several variables. There were no treatment effects on CL weight and diameter variation ($P \geq 0.78$), litter protein and DNA content variation ($P \geq 0.18$), litter variation in embryos length ($P = 0.83$), and litter protein : DNA ratio variation ($P = 0.18$) (Table 3). Litter Se concentration ($P < 0.05$) and total Se content variations ($P < 0.01$) were lower in $B_6$0 than in $B_6$10 gilts (Table 2). Additionally, $B_6$0 had lower litter weight variation than $B_6$10 gilts ($P < 0.05$) (Table 3).

These results also contrast with Fortier et al. (2012) in which litter total Se content variation in 30-d embryos was reduced in Se-supplemented animals comparing to CONT and no treatment effects was observed on litter weight variation. However, these authors have used 3.0 mg/kg of hydro-chloride pyridoxine in all diets, which is much lower than the 10.0 mg/kg supplementation ($B_6$10 gilts) used in the present study. According to Oka (2001), increased intracellular P-5-P level reduces the transcription of many hormones and anti-inflammatory factors, that are essential for embryos during the implantation period and placental development, such as glucocorticoids, progesterone, androgens, and estrogens. These changes in cell physiology potentially produce functional alterations at the systemic level (Fowden et al., 1998).

Dalto et al. (2014a) found very limited effects of B_6 supplementation (10.0 mg/kg) on 5-d porcine embryos transcriptome, showing that before placentation (hypoxic environment) this vitamin does not alter the porcine embryo antioxidant status and metabolic development. However, according to Combs Jr. (2012), the binding of P-5-P to hemoglobin enhances its O_2-carrying capacity, and considering that ROS production from O_2 occur naturally within cells, during any aerobic process, it is possible that the

rise in embryos oxygen tension caused by placentation, which causes a condition similar to the ischemia-reperfusion injury and is associated with higher expression of ROS markers (Jauniaux et al., 2000), might be potentiated by P-5-P leading to higher litter heterogeneity.

Conclusion

Although both Se levels and sources affected the long-term Se status of gilts, plasma GPX activity was influenced by B_6 supplementation. Early gestation appears to have important effects on blood Se, and plasma B_6 and protein concentrations.

The present results reinforce the peri-estrus period to be an adequate model to study treatment responses to oxidative stress conditions. Additionally, the level of 0.3 mg/kg of Se for gilts appears to be adequate to alleviate the oxidative stress induced by the peri-estrus period in the absence of hyperovulatory conditions.

The maternal Se transfer to porcine embryos occurs early in gestation and appears to be dependent upon the maternal Se status in MSe supplemented gilts. The normal (non-hyperovulatory) conditions and the high Se level in the basal diet might have attenuated the effects of Se supplementation on embryo development. However, the negative effects of B_6 on litter homogeneity deserve further investigations.

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Table 1. Composition of the basal diet (as-fed basis)[1,2].

Ingredients	Amount, %
Corn	52.6
Wheat shorts	20.0
Distillers dried grain with solubles.	10.0
Canola meal	9.7
Soybean hulls	4.0
Limestone	2.0
Salt	0.6
Monocalcium phosphate	0.5
L-Lysine	0.1
Choline	0.1
Feed curb[3]	0.1
Mineral and vitamin premix[3]	0.3

[1]The calculated compositions for ME, CP, lysine, Ca, and P of the basal diet were (as-fed basis) 2,702 kcal/kg, 14.0, 0.6, 1.0, and 0.6%, respectively.
[2]The basal Se and pyridoxine content of the diet were 0.3 mg/kg and 2.4 mg/kg, respectively (analytical values determined according to Giguère *et al.* (2005) and Matte *et al.* (2001), respectively).
[3]Inhibitor of mold growth
[4]Provided per kilogram of diet: Mn as manganous oxide, 40 mg; Zn as zinc oxide, 150 mg; Fe as ferrous sulfate, 140 mg; Cu as copper sulfate, 21 mg; I as calcium iodate, 2.0 mg; vitamin A, 14,580 IU; vitamin D, 1,500 IU; vitamin E, 44 IU; vitamin K, 2.6 mg; thiamine, 2.7 mg; riboflavin, 4.9 mg; niacin, 31 mg; pantothenic acid, 21 mg; folic acid, 10 mg; biotin, 400 µg; and vitamin B12, 25 µg.

Table 2. Selenium transfer to tissues and embryos, and embryo antioxidant status on d 30 of gestation, according to dietary Se treatments.

Item	CONT	MSe $B_6$0	MSe $B_6$10	OSe $B_6$0	OSe $B_6$10	SEM	a priori comparisons			
							CONT vs MSe + OSe	MSe vs OSe	$B_6$0 vs $B_6$10	Interaction effect
Liver Se concentration, µg/g	0.83	1.04	1.03	1.02	1.01	0.06	< 0.01	0.71	0.82	0.93
Kidney Se concentration[1], µg/g	3.45	3.74	3.79	3.70	3.87	0.15	0.26	0.26	0.26	0.26
CL Se concentration, µg/g	0.97	1.03	1.14	1.13	1.13	0.05	0.01	0.36	0.23	0.28
Litter total Se content, µg	6.55	8.00	7.66	7.99	7.55	0.71	0.13	0.93	0.58	0.94
Litter total Se content variation[2], %	12.82	12.77	19.71	13.94	18.42	1.89	0.12	0.98	< 0.01	0.52
Average embryo Se concentration, ng/g	291.33	351.35	353.01	389.89	382.11	15.86	< 0.01	0.04	0.85	0.77
Litter Se concentration variation[2], %	8.96	9.63	14.43	9.80	10.69	1.25	0.13	0.16	0.03	0.12
Average embryo GPX activity[3], U/mg of protein	68.50	69.34	72.84	67.76	69.64	4.73	0.80	0.61	0.57	0.86
Litter GPX activity variation[1], %	24.34	19.82	21.91	20.93	18.28	1.85	0.17	0.17	0.17	0.17

Results presented as the LS mean of 16 CONT, 17 MSe$B_6$0, 18 MSe$B_6$10, 17 OSe$B_6$0, and 16 OSe$B_6$10 gilts.
CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, MSe$B_6$0 = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), MSe$B_6$10 = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), OSe$B_6$0 = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), OSe$B_6$10 = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).
[1]According to the variance analysis, the effect of treatment was not significant ($P \geq 0.17$), therefore, it is not appropriated to accept the contrast analysis results.
[2]This value corresponds to the within-litter CV [(within-litter SD for the variable / within-litter mean for the variable) * 100].
[3]One unit (U) of glutathione peroxidase (GPX) activity is equal to 1 µmol of NADPH oxidized per minute.

Table 3. Corpora lutea (CL) and embryonic development on d 30 of gestation, according to dietary treatments.

Item	CONT	$MSeB_60$	$MSeB_610$	$OSeB_60$	$OSeB_610$	SEM	a priori comparisons			
							CONT vs MSe + OSe	MSe vs OSe	B_60 vs B_610	Interaction effect
Number of CL	19.4	19.27	19.07	17.75	18.19	0.77	0.34	0.12	0.88	0.68
Average CL weight, g	0.43	0.43	0.44	0.45	0.43	0.02	0.62	0.66	0.97	0.46
CL total weight, g	8.04	8.08	8.19	7.77	7.81	0.29	0.81	0.24	0.79	0.90
CL weight variation[1], %	14.69	15.32	16.11	14.80	11.78	1.78	0.93	0.18	0.53	0.29
Average CL diameter, mm	0.90	0.89	0.92	0.90	0.88	0.03	0.99	0.56	0.91	0.33
CL diameters variation[1], %	12.10	13.43	13.02	11.83	11.57	1.16	0.78	0.19	0.77	0.95
Number of viable embryos[2]	16.20	16.47	15.73	14.38	13.44	1.13	0.34	0.34	0.34	0.34
Embryo survival, %	84.30	85.55	81.66	80.70	73.77	4.94	0.49	0.20	0.28	0.76
Average embryo weight, g	1.41	1.40	1.38	1.43	1.41	0.05	0.96	0.56	0.72	1.00
Litter weight, g	22.75	23.05	21.63	20.61	20.12	1.81	0.50	0.28	0.60	0.80
Litter weight variation[1], %	8.41	7.56	10.90	8.99	12.69	1.46	0.33	0.27	0.02	0.90
Average embryo length, mm	21.97	22.32	21.95	22.28	22.08	0.33	0.61	0.88	0.38	0.78
Litter variation in embryos length variation[1], %	5.13	5.05	5.39	4.82	5.65	0.39	0.83	0.96	0.13	0.52
Average embryo protein content, mg	48.03	51.83	51.18	57.04	57.59	5.14	0.27	0.26	0.99	0.91
Litter total protein content, mg	774.51	843.08	817.97	846.42	823.87	105.81	0.63	0.97	0.82	0.99
Litter protein content variation[1], %	11.17	10.95	15.07	14.45	16.44	2.01	0.18	0.23	0.14	0.60
Average embryo DNA content, mg	4.91	4.78	5.02	4.98	5.01	0.16	0.85	0.53	0.39	0.50
Litter total DNA content, mg	79.38	78.68	77.68	72.25	69.26	6.16	0.48	0.23	0.75	0.87
Litter DNA content variation[1,2], %	16.82	14.74	15.979	12.68	15.41	1.27	0.12	0.12	0.12	0.12
Average embryo protein:DNA ratio, mg/mg	10.24	11.08	10.35	11.39	11.34	0.93	0.46	0.49	0.68	0.72
Litter protein:DNA ratio variation[1], %	21.69	17.90	16.65	14.87	12.93	4.02	0.18	0.40	0.69	0.93

Results presented as the LS mean of 16 CONT, 17 $MSeB_60$, 18 $MSeB_610$, 17 $OSeB_60$, and 16 $OSeB_610$ gilts.

CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

[1]This value corresponds to the within-litter CV [(within-litter SD for the variable / within-litter mean for the variable) * 100].

[2]According to the variance analysis, the effect of treatment was not significant ($P \geq 0.17$), therefore, it is not appropriated to accept the contrast analysis results.

Figure caption

Figure 1. Blood selenium concentration (ng/ml) of gilts on each estrus, presented as LS means ± SEM. CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, MSe = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), OSe = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY).
CONT < Se-suppl. (Se level, $P < 0.01$); MSe < OSe (Se source, $P < 0.01$); treatment X times interaction ($P < 0.01$).

Figure 2. Plasma GPX Activity (mU/mg Hb) of gilts for each estrus and at 30 d of gestation, shown as the LS means ± SEM of $MSeB_60$ + $OSeB_60$ (B_60) and $MSeB_610$ + $OSeB_610$ (B_610).
$MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).
$B_60 > B_610$ (B_6 level, $P < 0.01$).

Figure 3. Blood selenium concentration (ng/ml of blood) of gilts at fourth estrus, shown as LS means ± SEM. Day 0 was the day of physiological estrus.
CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, MSe = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), OSe = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY).
CONT < Se-suppl. (Se level, $P < 0.01$); MSe < OSe (Se source, $P < 0.01$); time effect ($P < 0.05$).

Figure 4. Plasma P-5-P concentrations (μM) of gilts at fourth estrus, shown as LS means ± SEM. Day 0 was the day of physiological estrus.

CONT = basal diet containing 0.3 mg/kg and 2.4 mg/kg of natural Se and pyridoxine respectively, $MSeB_60$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO), $MSeB_610$ = basal diet supplemented with 0.3 mg/kg of sodium selenite (Sigma-Aldrich, St. Louis, MO) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA), $OSeB_60$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY), $OSeB_610$ = basal diet supplemented with 0.3 mg/kg of Se-enriched yeast (Alltech Inc., Nicholasville, KY) and 10 mg/kg of hydro-chloride pyridoxine (P9755 Sigma-Aldrich, St-Louis, MO, USA).

$B_60 < B_610$ (B_6 level, $P < 0.01$); $MSeB_610 >$ others (Se source X B_6 level interaction, $P < 0.01$).

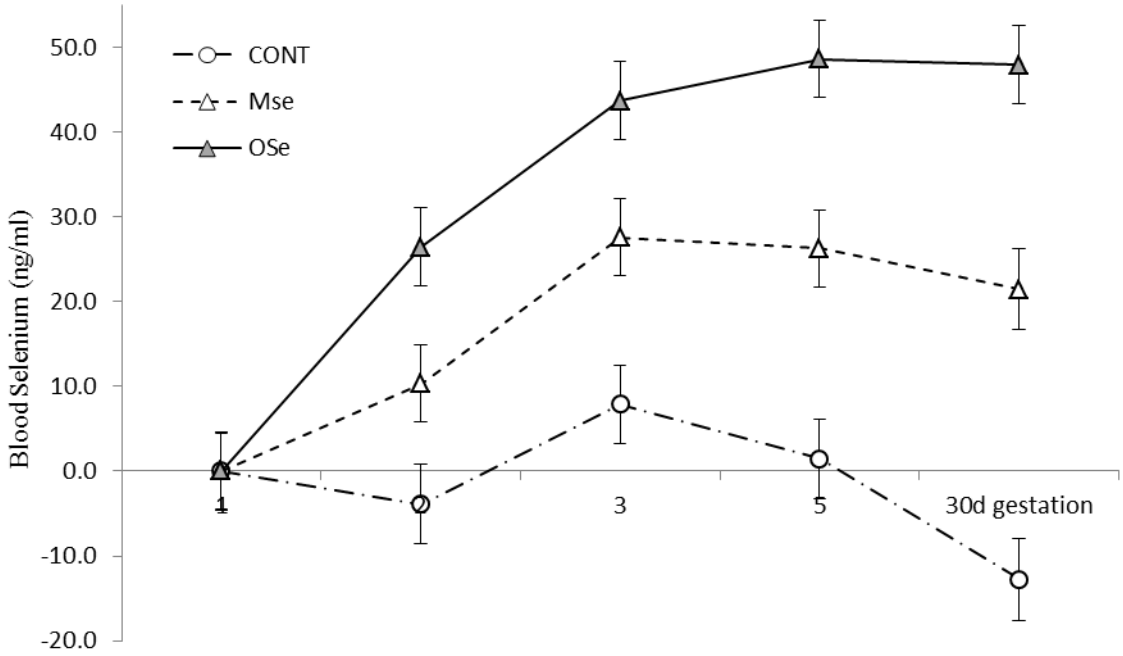
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
Blood Selenium (ng/ml)
CONT
Mse
OSe
1
3
5
30d gestation

Figure 1

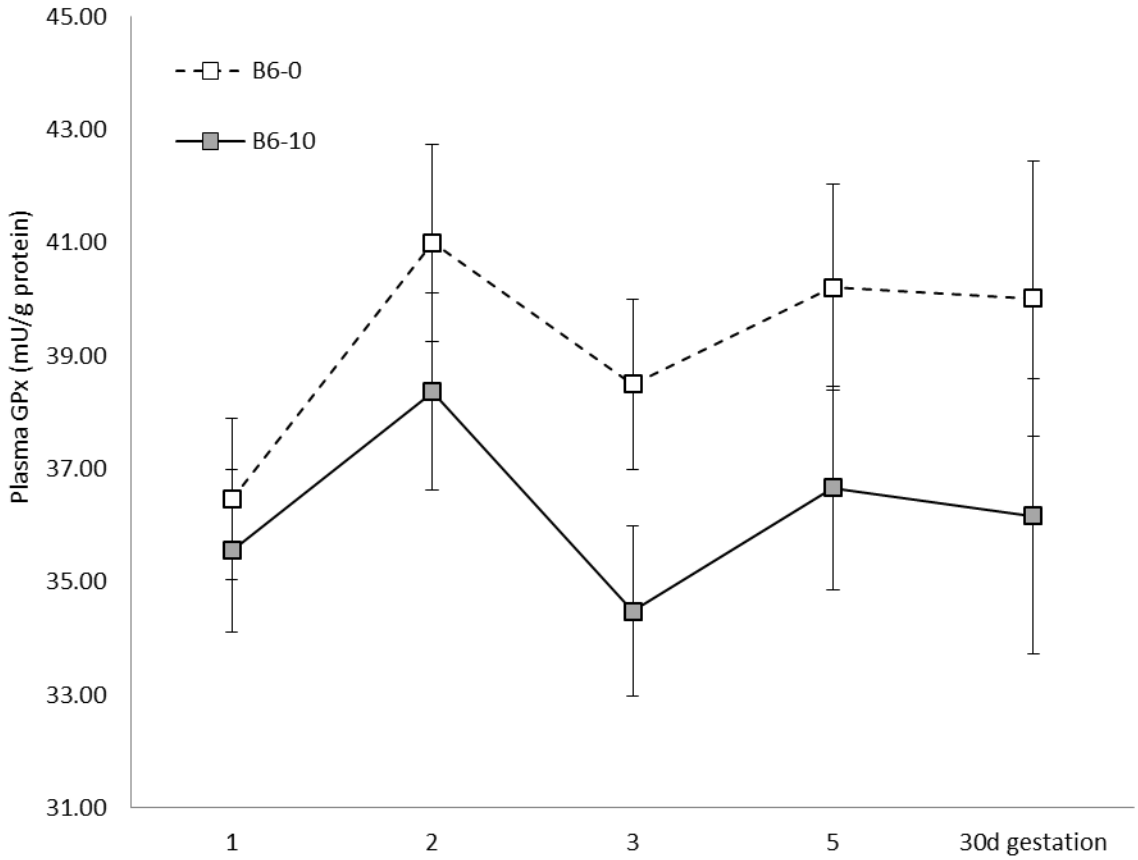
45.00
43.00
41.00
39.00
37.00
35.00
33.00
31.00
Plasma GPx (mU/g protein)
B6-0
B6-10
1
2
3
5
30d gestation

Figure 2

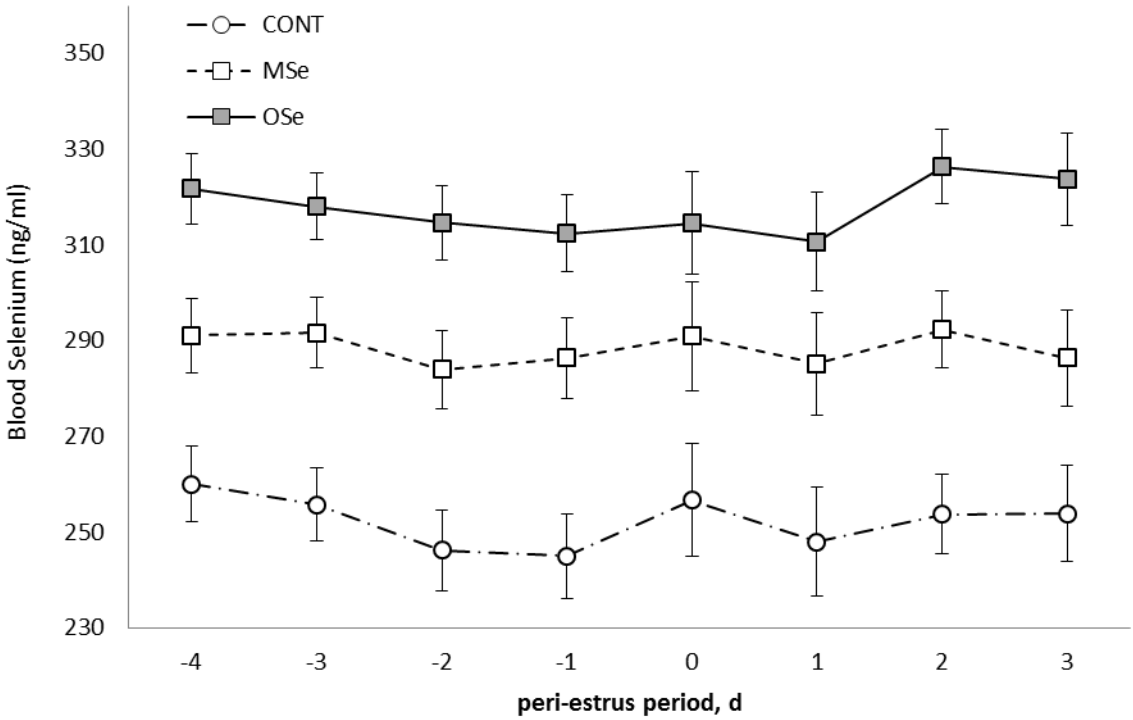
CONT
MSe
OSe
Blood Selenium (ng/ml)
350
330
310
290
270
250
230
-4
-3
-2
-1
0
1
2
3
peri-estrus period, d

Figure 3

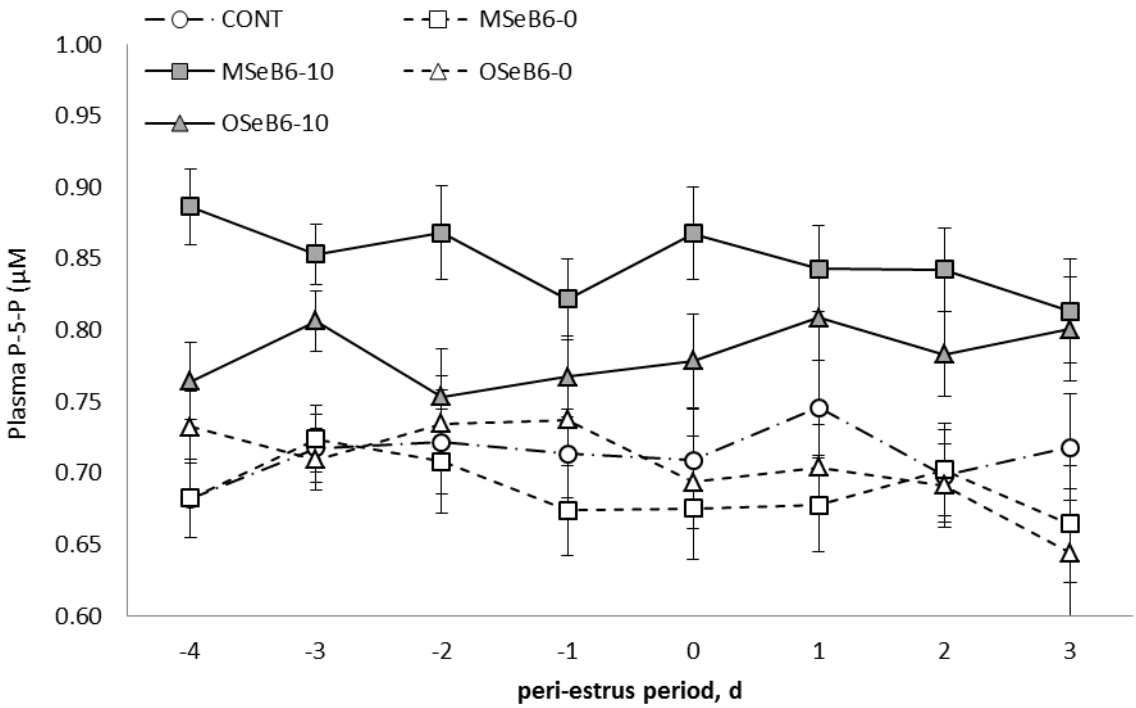
CONT
MSeB6-0
MSeB6-10
OSeB6-0
OSeB6-10
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
Plasma P-5-P (µM
-4
-3
-2
-1
0
1
2
3
peri-estrus period, d

Figure 4

6. SUPPLEMENTAL INFORMATION

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