THE ROLE OF GLUCOSINOLATES AND IODINE ON THYROID HORMONE CONCENTRATIONS IN MARES AND FOALS

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Large Animal Clinical Sciences

University of Saskatchewan

Saskatoon

By

MARIA FERNANDA LOPEZ RODRIGUEZ

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Large Animal Clinical Sciences
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan [postal code] Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

ABSTRACT

Effect of Sinigrin and a Low Iodine Diet on Serum Iodine and Thyroid Hormone Concentrations in Non-pregnant Mares and Serum, Colostrum and Milk Iodine Concentrations in Postpartum Mares and Foals

Adequate levels of thyroid hormones and iodine are essential for health and normal fetal development. Disruptions in maternal iodine metabolism during pregnancy or transfer of iodine to the fetus may cause fetal thyroid dysfunction, which may lead to severe negative effects on development and organ maturation. A syndrome characterized by prolonged gestational length, moderate to severe musculoskeletal deformities such as contracted tendons, failure of tarsal and/or carpal ossification, prognathia, signs of dysmaturity and goiter called congenital hypothyroidism dysmaturity syndrome in foals (CHDS) has been described. The risk factors for CHDS include a lack of trace mineral supplementation and consumption of *Brassicaceae* family plants containing glucosinolates (GSL) during pregnancy. Glucosinolates have negative effects on thyroid function and animal health in other species. There are no previous reports on the effects of GSL on thyroid function and iodine levels in horses at any age and physiologic state. There is also a lack of robust information on iodine levels in mares and foals in different body fluids and secretions despite the prevalence of CHDS in foals in Western Canada, where *Brassica* family GSL plants are widely cultivated, mustard family GSL containing weeds are abundant and soil iodine concentrations are inadequate.

We hypothesized that the combination of a low iodine and a glucosinolate (GSL) (sinigrin) containing diet fed for 3 months to non-pregnant mares would affect thyroid hormones and serum iodine concentrations in non-pregnant mares and that colostrum, milk and serum iodine levels and thyroid hormone concentrations would decrease over time in healthy post-partum mares and foals.

Nineteen mares aged 2 to 14 years were divided into Control with iodine supplementation and, Low (20 mmol/day) and High GSL (35 mmol/day) diet groups without iodine supplementation. Thyrotropin releasing hormone (TRH) stimulation tests and serum iodine measurements were performed at 0 and 12 weeks. Total triiodothyronine (TT3), total thyroxine

(TT4) and thyroid stimulating hormone (TSH) concentrations at baseline and in post-TRH samples were measured. The post-TRH fold changes (FC) were calculated for TSH, TT3 and TT4. There was a group and group by week interaction (P <0.001) in TT4 FC values, with week 12 Control concentrations higher (P < 0.006) than all groups. Iodine concentrations decreased (P < 0.002) in GSL mares.

Additionally, colostrum, milk and blood samples from ten draft mares and foals, with an estimated intake of 39 mg iodine per day per mare during pregnancy were obtained at foaling date and 10 days later. Measurements included: mare basal concentrations of serum: TT3, TT4, iodine; iodine in colostrum at day 0 and milk iodine (day 10); foal basal TT3, TT4 and serum iodine (day 0 and 10). Median ± SE foal serum iodine (268.5 ± 7.6 µg/L), TT4 (1225 ± 47.8 nmol/L) and TT3 (14.2 ± 1.1 nmol/L) at foaling date were higher than at 10 days (70 ± 3.6 µg/L; 69.6. ± 20.4 nmol/L; 5.4 ± 0.3 nmol/L, respectively). Colostrum iodine levels (165 ± 15.1 µg/L) were higher than milk (48 ± 5.6 µg/L) levels.

In conclusion, the effect of sinigrin along with a low iodine supplemented diet did not have a negative impact on body weight and overall health in non-pregnant mares, but T4 responsiveness and iodine levels decrease after 12 weeks. Similar controlled studies on the effects of GSL should be performed in pregnant mares in which thyroid function and adequate levels of iodine are more critical for the developing fetus. Furthermore, nutritional studies with known and confirmed iodine intakes should be performed to determine robust reference ranges for iodine levels in serum, colostrum and milk from mare and foal pairs at different ages.

Keywords: glucosinolates; singrin; iodine; thyroid hormones; milk; colostrum

ACKNOWLEDGMENTS

I would like to acknowledge the guidance and expertise received from my supervisor, Dr. Claire Card, and my Advisory Committee members, with special gratitude to Dr. Nadia Cymbaluk and Bernard Laarveld. Thanks to Hilltopper Clydesdales of Saskatoon, SK and for their participation in this research by providing the serum, colostrum and milk samples from mare and foal pairs.

I would also like to thank Dr. Tasha Epp for the statistical assistance throughout the project.

I am also grateful for the exceptional help from Carolina Serrano and Mikhaela Trasher, whose assistance in taking the serum, colostrum and milk samples from mare and foals, as well as during the feeding trial was essential; for the laboratory assistance from Mrs. Susan Cook and Paula Mason, as well as the amazing summer students: Jasmine Paulson, Mikayla Swirski, Brandi Bakken and Ashlyn Ketterer for their exceptional help and support.

Finally, thanks to the financial support from the Mark and Pat DuMont Equine Research Fund of the Western College of Veterinary Medicine, and the Saskatchewan Agriculture Development Fund.

DEDICATION

I dedicate my work to my parents and my sister to whom I am grateful for all the support and guidance they have provided in every single step of my life. I am grateful to my mom, who has always been by my side in every decision, success and failure in life and who received several phone calls at 2 am in the morning because I was feeling sad and homesick and always had the right words to make me feel better.

I also thank my sister, whose emotional and physical strength has taught me that there are no limits, and to my friends and colleagues: Karen Pimentel, Nora Chavarria, Mikhaela Trasher, Paula and Calvin Mason, Ronan Chapuis, Carolina Serrano, my best friends: Elisa, Fer and Cintya thank you for your support.

To Dr. Claire Card, who played a key role in my professional training and without her I wouldn't have learned how to drive. I will always be grateful for all the patience, support and guidance.

TABLE OF CONTENTS

CHAPTER 3 - EFFECT OF THE GLUCOSINOLATE SINIGRIN IN COMBINATION WITH A NON-IODINE SUPPLEMENTED DIET ON SERUM IODINE AND THYROID HORMONE CONCENTRATIONS IN NON-PREGNANT MARES

CHAPTER 4 - A FIELD STUDY OF SERUM, COLOSTRUM, MILK IODINE AND THYROID HORMONE CONCENTRATIONS IN POSTPARTUM DRAFT MARES AND FOALS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

cAMP	Cyclic 3', 5'- adenosine monophosphate
BW	Body weight
CHDS	Congenital hypothyroidism dysmaturity syndrome
D1	Deiodinase I
D2	Deiodinase II
D3	Deiodinease III
DIT	Diiodotyrosine
DM	Dry matter
eCG	Equine chorionic gonadotropin
EMS	Equine metabolic syndrome
FC	Fold change
fT4	Free thyroxine
fT3	Free tri-iodothyronine
fT4d	Free thyroxine by equilibrium dialysis
fT3d	Free tri-iodothyronine by equilibrium dialysis
FNA	Fine needle aspiration
g	Gram
GEE	Generalized estimated equation
GSL	Glucosinolates
hCG	Human chorionic gonadotropin
THs	Thyroid hormones
HPA	Hypothalamo-pituitary-adrenal
HPT	Hypothalamo-pituitary-thyroid
HR	Heart rate
ICP-MS	Inductively coupled plasma mass spectometry

I^-	Iodide
kg	Kilogram
L	Liter
L-T4	Levothyroxine sodium
LC-MS/MS	Liquid chromatography with tandem mass spectometric
MIT	Monoiodotyrosine
MRLS	Mare reproductive loss syndrome
mL	Milliliter
mmol	Millimole
µg	Microgram
NIS	Sodium/Iodide symporter protein
Na^+	Sodium
OMP	Oriental mustard powder
ppb	Parts per billion
ppm	Parts per million
PTU	Propylthiouracil
RSM	Rapeseed meal
RR	Respiratory rate
SE	Standard error
TH-MSD	Thyroid musculoskeletal disease
TPO	Thyroid peroxidase
T4	Thyroxine
T3	Triiodothyronine
TT4	Total thyroxine
TT3	Total triiodothyronine
TRα	Thyroid hormone receptor alfa
TRβ	Thyroid hormone receptor beta
TSH	Thyroid stimulating hormone
TRH	Thyroid releasing hormone

CHAPTER 1: HYPOTHESES AND OBJECTIVES

Null Hypotheses and Objectives

Experiment 1: Feeding a non-iodine supplemented diet and the glucosinolate (GSL) sinigrin for 12 weeks has no effect on serum iodine and thyroid hormone levels in non-pregnant mares.

Objectives:

1) To determine the effect of feeding a non-iodine supplemented diet and sinigrin on total thyroxine (TT4), total tri-iodothyronine (TT3), and thyroid stimulating hormone (TSH) levels at 0 and 12 weeks later
2) To determine the effect of feeding a non-iodine supplemented diet and sinigrin on the response to thyroid releasing hormone (TRH) at 0 and 12 weeks later
3) To determine the effect of feeding a non-iodine supplemented diet on serum iodine concentrations in non-pregnant mares at 0 and 12 weeks later

Experiment 2: Serum, milk and colostrum iodine levels from healthy post-partum mares are unrelated to their foals thyroid hormone concentrations and serum iodine levels in the first 10 days post foaling. The equine mammary gland does not concentrate iodine for the newborn foal.

Objectives:

1) To determine the level of iodine in mares' serum, milk and colostrum following a healthy pregnancy
2) To determine the level of foals' serum iodine in relation to TT4 and TT3 concentrations at foaling day and 10 days later following a healthy pregnancy
3) To determine if there is a relationship between iodine levels in mare's colostrum and milk with foal's serum iodine levels.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

It has been demonstrated that healthy thyroid function is essential for metabolism, and reproduction; and impacts seasonal reproductive activity, estrus cyclicity, fetal programming and fetal development in horses [1–3]. Thyroid function, because of its central role in fetal development, post-natal growth and metabolism affects the future performance of horses [1]. The prevalence and impact of thyroid dysfunction in the equine population has not been properly assessed and there are still many knowledge gaps [4]. There are a few studies that have examined the impacts of environmental factors such as toxic weeds, low environmental iodine and excess nitrates on pregnancy outcomes in mares [5–9]. Early pregnancy loss, abortion, goitre and musculoskeletal defects have been reported in association with these exposures. Recently Swerczek and Dorton (2019) published information that implicated high nitrate levels as a cause of neonatal thyroid dysfunction, limb contracture and mare reproductive loss syndrome (MRLS) in Kentucky [9].

The experiments in this thesis address areas where there is little information, such as the impact of glucosinolates (GSL) on thyroid function in non-pregnant mares and a description of serum, colostrum and milk iodine levels in post-partum mares in relationship to thyroid hormone levels. In preparation for this research, a general review of equine: thyroid anatomy, physiology, role of iodine in thyroid function, assessment of thyroid function and dietary factors affecting thyroid function in non-pregnant mares, pregnant mares, and foals; and the role of thyroid hormones and iodine metabolism in fetal growth, maturation, and during the post-partum period was performed. Furthermore, a review of the principal nutritional factors influencing thyroid function in horses, such as GSL, and their effects on serum iodine and thyroid hormone levels in non-pregnant mares compared to other domestic species was performed.

2.2 Anatomy

During fetal development, the two principal embryonic structures involved in thyroid gland development are the thyroid bud, which originates from the endoderm of the primitive

larynx and is responsible for the follicular cell development; and the ultimobranchial body, which gives rise to the calcitonin cells and originates from the fourth pharyngeal pouch [10]. Furthermore, the pre-colloid, colloid and follicular stages of the thyroid gland development are essential for growth and early maturation of the fetal thyroid gland, resulting in the functional development of the fetal hypothalamic-pituitary-thyroid (HPT) axis, which is capable of secreting thyroid hormones when the follicular stage is fully developed [11].

In the adult horse, the thyroid gland is located in the dorsolateral aspect of the third to sixth tracheal rings and consists of two lobes that are connected by an isthmus of fibrous tissue. It has been reported to have a normal mean (range) weight of 0.08 g/kg (0.01 – 0.15 g/kg) for adult horses and 0.28 g/kg (0.12 – 0.67 g/kg) for foals, based on grams of thyroid tissue per kilogram of body weight calculation[12]. The weight and size of the thyroid gland was also reported by Schotthauer (1931) who reported that the thyroid weighed about 23 grams (g) for adult horses with a mean weight of 0.04 g/kg and a range of 0.02 -0.06 gm/kg. Thyroids greater than 0.066 g/kg were histologically abnormal [13]. The dimension of the thyroid lobes measured using transcutaneous ultrasound were: length 3.6 – 3.9 cm, width 1.9 – 2.1 cm and height 1.6 - 1.7 cm [14,15].

The vascular supply enters the thyroid lobe at the cranial pole with the cranial thyroid artery from the external carotid and at the caudal pole with the caudal thyroid artery from the subclavian artery, respectively [16]. The gland is not easily visible nor palpated in the healthy young horse; however, in horses with thyroid gland tumors or goiter these may be palpated as a firm and movable structure of variable size [10]. Histologically, the thyroid gland is surrounded by a capsule of connective tissue and divided into lobules; each lobule contains multiple follicles filled with colloid. The appearance of the follicular cells changes according to the thyroid gland activity, and ranges from low cuboidal cells in the resting stage to cuboidal or columnar cells with vacuoles in the active stage [17]. The thyroid follicle is considered to be the functional unit of the thyroid gland, whereas, the colloid contains an essential protein for thyroid hormone synthesis and storage, called thyroglobulin [18]. In addition, the thyroid gland also contains parafollicular or C cells which play a key role in regulating follicular cell activity [19].

2.3 Physiology of the equine thyroid gland

2.3.1 General physiology

Thyroid hormone synthesis involves two major actions, entry of iodide (I^-) to the thyroid and into follicular cells through the sodium / iodide (Na^+/I^-) symporter, where I^- is oxidized into iodine by the thyroid peroxidase enzyme. Thyroglobulin, a glycoprotein containing multiple tyrosine residues, is synthesized in the follicular epithelial cells, and secreted into the follicular lumen where it is stored as colloid. Once in the follicular lumen, iodine binds to carbon position 3 or 5 of multiple tyrosine residues present in the thyroglobulin to form monoiodotyrosine (MIT) and diiodotyrosine (DIT), which are then coupled by the enzyme thyroid peroxidase to form thyroxine (T4) and triiodothyronine (T3). Both TT3 and TT4 are stored in the follicular lumen with the remaining thyroglobulin, and the MIT and DIT residues that did not couple [18,19]. Secretion of thyroid hormones into circulation involves endocytosis and degradation of the colloid containing the thyroglobulin followed by the release of greater amounts of T4. Despite higher concentrations of T4 in plasma, T3 is the biologically active hormone. Triiodothyronine's binding capacity to the thyroid hormone receptor in target tissues is reported to be 15-fold higher than T4, and T3 is not highly coupled to plasma proteins compared to T4. However, T4 has a lower metabolic clearance rate with a half-life of approximately 7 days compared to the one-day half-life of T3. The ability of T4 to bind to plasma proteins such as thyroxine binding globulin, serves as a circulating reserve to ensure adequate deliver of T3 to target tissues based on their metabolic rate [18,20]. Before thyroid hormones (THs) are released into circulation, a specific amount of T4 undergoes local deiodination to form T3 in the thyroid, this serves as a recycling mechanism of iodine for further involvement in the new synthesis of thyroid hormone, whereas the majority of the circulating T3 is produced by the deiodination of T4 in peripheral tissues. The process consists of removal of iodine from carbon 5 of the T4 outer ring and is regulated by the action of deiodinases type I, II and III (D1, D2 and D3) [18]. The D1 enzyme is found mainly in the liver, kidney and thyroid gland, and is involved in the production of the majority of the circulating T3. Similarly, D2 is expressed mainly in the brain, pituitary gland, adipose tissue and thyroid gland; it is responsible for controlling intracellular T3 concentrations and protects the tissues from the negative effects of low thyroid hormone concentrations for a long period of time by allowing efficient local conversion of T4 to T3 in tissues that are more susceptible to these

detrimental effects, such as the brain. In contrast, D3 is responsible for inactivation of T4 by converting it to reverse T3 (rT3). The D3 is predominantly found in brain, placenta and skin, and plays a key role in placental protection of the fetus against exposure to high levels of maternal thyroid hormones [18,20,21].

It is worth mentioning that thyroid hormone bioavailability not only depends on the expression and activity of transporters, but also on the expression of intracellular THs receptors in the target tissues. Thyroid hormone receptors belong to the ligand dependent transcription factor superfamily. Thyroid receptor alpha (TRα) and thyroid hormone receptor beta (TRβ) are the two isoforms reported to be expressed in the fetus by mid-gestation [11]. TRα is expressed in almost all the tissues, but their effects have been studied more in the liver, bone, fat, brain and heart. The different expressions of both isoforms are well investigated in the developing brain. In rats, TRα is highly expressed in the brain during fetal development, whereas TRβ is highly expressed in the brain in the postnatal development [22]. In addition, TRα expression is highly dominant in the cardiovascular system. Binding of thyroid hormone increases heart rate and cardiac output [22]. In domestic animals, there are species specific expressions between the two isoforms. For example, in fetal pigs, TRβ is highly expressed in heart and skeletal muscles at birth, whereas TRα expression decreases [11].

The HPT axis controls and regulates the synthesis and release of T4 and T3 through a negative feedback mechanism. Thyrotropin releasing hormone (TRH) is synthesized by the hypothalamic paraventricular nucleus and is transported to the adenohypophysis through the capillary plexus, where it binds to receptors on adenohypophyseal cells called thyrotrophs. Thyrotrophs then release thyroid-stimulating-hormone (TSH), also referred to as thyrotropin, into the systemic circulation [20]. The TSH is transported to the thyroid gland and binds to TSH receptors located in thyroid follicular epithelial cells, and acts to stimulate iodine uptake and thyroid hormone synthesis. It is worth mentioning that growth and function of the thyroid gland is highly stimulated by the intracellular signals caused by TSH, such as the rise in cyclic 3', 5'-adenosine monophosphate (cAMP), for that reason a persistent increase in circulating TSH levels above the normal range will result in marked thyroid hyperplasia [18,20]. The negative feedback is based on the local production of T3, which acts directly on thyroid hormone receptors and inhibits the hypothalamic and pituitary synthesis and secretion of TRH and TSH, respectively.

For that reason, elevated thyroid hormone concentrations in blood inhibit TSH secretion, whereas low levels of thyroid hormone enhance the release of TSH [18,21].

2.3.2 Effect of age, sex and geographic location

The fetal hypothalamic-pituitary-adrenal (HPA) axis undergoes developmental changes that leads to the final secretion of cortisol. Little is known regarding the effects of cortisol on equine thyroid hormone levels during the last 48 to 72 hrs of pregnancy in the mare; however, the prepartum increase of fetal cortisol seen in sheep causes an abrupt increase in circulating T3 by an increase in T4 deiodination. In addition, high T3 concentrations are believed to be dependent of the proper maturation of the HPA axis [23]. In the neonatal period, thermogenesis, adequate pulmonary exchange, hepatic gluconeogenesis and post-natal maturation of vital organs and systems are essential for neonatal survival and adequate adaptation [11]. Levels of TT4 and TT3 are approximately 14 times higher in the healthy neonatal foal compared to adult horses. High rates of peripheral conversion of T4 to T3 have been reported. High thyroid hormone levels coincide with a high metabolic demand in the neonatal period followed by a dramatic decrease in the first week of life and then a gradual decline over the next months of life until thyroid hormones levels are similar to the adult levels [11,24].

In contrast, resting T4 levels decrease, whereas TSH levels increase as the horse ages, similar to what has been reported in elderly humans and dogs. It is still uncertain whether the increase in circulating levels of TSH noted in older horses indicates that subclinical hypothyroidism is present as a consequence of a higher proportion of older horses having abnormal thyroid tissue, or is a normal hormonal change in the aged horse [25]. Other factors such as gender and weaning have been reported to have a direct effect on thyroid hormone levels in the growing foal. Fazio et al. (2007) reported an abrupt decrease in TT3 levels without significant changes in TT4 around the time of weaning suggesting the ability of the thyroid gland to adapt to different physiological processes in the first year of life [26]. Furthermore, growing colts had higher basal TT4 levels around weaning time (5 months) and 13 months of life, with a positive correlation between basal T3, body weight ($r = 0.240$; $P < 0.02$) and age ($r = 0.281$; $P = <0.003$) compared to fillies [26].

The ability of the horse to adapt to endemic goiter areas has been partially assessed in North-eastern Sicily, Italy, where severe iodine deficiency disorders have been reported in humans, including thyroid dysfunction. There were no significant differences in resting THs levels between horses managed in endemic goiter areas versus non-endemic areas. There were only significant differences between male and female groups, and it was concluded that there was a physiological adaptation response to the low iodine environment, as there were no signs of hypothyroidism in horses compared to what was reported in humans exposed to iodine deficient environments. Nevertheless, TSH levels were not measured in all horses, thus, complete thyroid function was not fully assessed [27].

2.3.3 Thyroid function in pregnant vs non-pregnant mares

In all mammalian species, metabolic and hormonal changes during pregnancy have a direct effect on maternal thyroid hormone synthesis. Maternal HPT axis maintains adequate levels of THs based on metabolic and energy demands from the dam and the growing conceptus. In women, the human chorionic gonadotropin (hCG) secreted during the first trimester of pregnancy is not only essential for maintenance of pregnancy, but also has thyrotropic effects. The alpha subunit of TSH is reported to be structurally similar to hCG, which induces an abrupt increase in circulating free T4 (fT4) and free triiodothyronine (fT3). TSH levels are commonly low due to the negative feedback caused by high THs levels [28]. It has been demonstrated in humans, and speculated in domestic animals that during the first trimester of pregnancy, the fetus relies entirely on maternal THs due to the inability of the fetal HPT axis to synthesize THs [29]. Thus, maternal THs play a key role in early embryonic growth and development. In addition, an increase in circulating TT4 and TT3 is accompanied by an enlargement of maternal thyroid gland of approximately 18% and an increase of 50% in TH production, demonstrating a high degree of thyroid gland activity in pregnant women [28]. In contrast, Fazio et al (2016) reported no significant differences in serum TT4 and TT3 between pregnant and non-pregnant mares over a 12-month period. Nevertheless, higher TT3 circulating levels were observed on the second month of pregnancy, and the lowest in the 11th month, whereas, TT4 circulating levels were higher in the 5th month and decrease by the 11th month [30]. Similarly, TT3 has been reported to change over time during the first 12 weeks of pregnancy, with no effect on TT4. Higher TT3 circulating levels were reported in the 7th and 12th week of gestation compared to non-pregnant

mares, which also coincides with an increase in equine chorionic gonadotropin (eCG) secretion by the endometrial cups, indicating that there is an effect of pregnancy on thyroid hormone and also a period of high synthesis and release of maternal THs during early pregnancy [31,32].

2.3.4 Thyroid hormone values in non-pregnant mares and foals.

Reference ranges from the Animal Health Diagnostic Center at Cornell University, were used for the present study. These values were based on samples obtained from healthy horses. Adult reference ranges are:

- T3: 0.408 – 1.088 nmol/L
- T4: 12.87 nmol/L – 38.6 nmol/L
- fT4: 15.45 pmol/L - 23.17 pmol/L

Foal reference ranges in the first 14 days of age are reported in Table 2.1, showing the dramatic decrease in THs concentrations after 7 days post-foaling.

Table 2.1 Mean (range) of serum levels of total thyroxine (TT4 nmol/L) and triiodothyronine (TT3 nmol/L in foals from foaling date (day 0) to 14 days post-foaling (Animal Health Diagnostic Center- Cornell)

Age (Days)	T4 nmol/L	T3 nmol/L
0	392.5 (339.2 - 445.7)	6.6 (4.4 - 8.7)
1	319.2 (275.0 - 363.3)	3.395 (1.2 - 5.6)
2	348.1 (237.6 - 448.3)	11.6 (7.0 - 16.7)
3	276.2 (228.2 - 329.3)	11.9 (4.7 - 17.9)
4	281.9 (221.9 - 349.8)	9.6 (6.9 - 12.3)
5	187.9 (166.1 - 217.9)	6.3 (3.4 - 10.5)
6	210.8 (134.4 - 337.4)	7.7 (3.8 - 13.7)
7	140.8 (77.6 - 193.2)	6.8 (5.4 - 9.7)

13	46.9 (30.9 - 62.9)	3.2 (2.8 - 3.4)
14	71.8 (46.8 - 99.7)	3.5 (3.05 - 4.42)

It is worth mentioning that there are other authors who reported normal reference ranges that can be useful when assessing thyroid hormone levels. Breuhaus (2018) reported serum TT4, TT3, and free T4 and T3 by equilibrium analysis (fT4D, fT3D), and TSH levels in 24 to 36-hour neonatal healthy foals, and young (3 - 6 years and 7 – 10 years), middle aged (11 – 14 years) and old horses (≥ 15 and ≥ 20 years). The most relevant information is the decrease in TT4 concentrations as the horses age compared to younger animals. The hormone values are shown in Table 2.2 [24,25].

Table 2.2. Median ± 95% confidence interval and range of serum levels of total thyroxine (TT4 nmol/L), free thyroxine (fT4 pmol/L), total triiodothyronine (TT3 nmol/L), free triiodothyronine (fT3 pmol/L) and TSH (ng/mL) in foals, and young, middle age and old horses.

Age	n	TT4 nmol/L	fT4D pmol/L	TT3 nmol/L	fT3 pmol/L	TSH ng/ml
24. – 36 hrs	20	260.5 ± 16.9	97.5 ± 13.6	8.2 ± 0.8	10 ± 2.	0.24 ± 0.07
		(191 – 318)	(49 – 144)	(6 – 11.8)	(5.6 – 24.2)	(0.10 – 0.68)
3 - 6 yrs	15	24 ± 5.8	23 ± 4.4	1.2 ± 0.22	1.7 ± 0.5	0.3 ± 0.08
		(8 – 44)	(9 – 40)	(0.6 – 2.0)	(1.2 – 4.2)	(0.03 – 0.42)
7 – 10 yrs	27	18 ± 3.6	19 ± 3.7	0.8 ± 0.22	1.5 ± 0.5	0.4 ± 0.08
		(6 – 46)	(9 – 47)	(0.4 – 2.9)	(0.5 – 5.7)	(0.06 – 0.80)
11 – 14 yrs	13	19 ± 4.3	16 ± 7.0	0.9 ± 0.23	1.3 ± 0.8	0.3 ± 0.09
		(8 – 32)	(7 – 40)	(0.4 – 1.6)	(0.8 – 4.5)	(0.03 – 0.5)
≥ 15 yrs	16	15 ± 4.5	24 ± 3.2	0.9 ± 0.2	2.05 ± 0.8	0.4 ± 0.13
		(6 – 32)	(11 – 33)	(0.3 – 1.8)	(0.1 – 5.9)	(0.1 – 0.97)
≥ 20 yrs	10	14.5 ± 6.8	23.5 ± 4.4	0.95 ± 0.2	2.05 ± 1.15	0.5 ± 0.2
		(6 – 32)	(11 – 32)	(0.4 – 1.4)	(0.1 – 5.9)	(0.2 – 0.97)

2.3.5 Effect of seasonality

The link between thyroid hormone levels, seasonality and temperature have been studied in many domestic animals whose reproductive performance relies solely on the photoperiod and the availability of the feed under natural circumstances, like the horse and small ruminants. Hormonal levels, energy utilization and metabolic rate tends to be different in a seasonal animal versus a non-seasonal animal, hence, thyroid hormone levels may have variations during the year. It is not clear if there are interactions between seasonality and ambient temperature in horses and what those effects might be on thyroid hormone levels. Johnson (1986) reported higher serum TT4 levels between October and November, with a very slight negative correlation with photoperiod; higher prolactin concentrations from May to August were found with lower levels from September to February, whereas higher TT3 levels were found to be in December through May and lower levels from July to October. Mares in the previous study were exposed to ambient photoperiod consisting of 8 hours light and 16 hours dark in the winter period. The study results showed that prolactin levels were positively correlated with photoperiod. Prolactin was lower in winter months and inversely related to THs concentration. There was an increase in TT4 and TT3 during the winter months, suggesting an increase in metabolic rate around this time; TT3 was reported to be inversely correlated with temperature suggesting that lower temperatures in winter enhances an increase in circulating TT3 [33]. The relationship of THs levels and season has also been reported in goats. A study conducted in Nigeria, where TT3 and TT4 levels were measured in cold-dry and hot dry weather, revealed an increase in THs levels during the cold dry months demonstrating an increase in metabolic rate along with an increase in heat generation to promote optimal body temperature during low ambient temperature, similar to what it has been reported in other species such as goats [34]. On the contrary, Fazio et al (2018) reported higher T3 levels in May and June and the lowest in February, whereas T4 levels were higher in July to August and lower in February to March in mares housed in breeding farms near Catania, Sicily. It is probable that the differences reported in Fazio's study could be due to the geographic area and the temperature during summer and winter months [30]. Critical studies in this regard concerning seasonal and temperature need to be performed.

2.4 Role of iodide in thyroid function

2.4.1 Iodide metabolism in the mare

There are few studies on iodine metabolism in the horse, and it has been assumed that it is similar to what has been reported in humans. There are two main sources of iodine: plants grown on iodine-rich soil and seawater, seaweed or alga phytoplankton which have also been reported to be a source of iodine in Asian countries. It has been reported that grass forage provides approximately 0.2 – 0.3 mg I^- per kg of dry matter (DM) [35]. After ingestion, iodide is 100% bioavailable and is fully absorbed in the small intestine, mainly in the duodenum. Once in the bloodstream, I^- metabolism involves three steps: the first step starts with the uptake of I^- from bloodstream into the follicular cell of the thyroid gland. This process is accomplished by an active transport system through the sodium/iodide symporter protein (NIS) found in the follicular cell, it is Na^+-K^+ pump dependant and its activity is regulated by TSH signaling and circulating iodine levels. It is worth mentioning that the NIS has the capacity to raise intrathyroidal levels of iodide 20-50-fold higher than in general circulation. The NIS is also present in mammary gland tissue, salivary glands, gastric and intestinal mucosae. Furthermore, I^- transport through this symporter can be substituted by other substances such as perchlorate, nitrate, thiocyanate and pertechnetate causing a decrease in I^- uptake by the thyroid gland [36]. In humans, it has been demonstrating that NIS in the lactating mammary gland provides an important supply of iodine to the newborn [36]. The second step involves the synthesis and secretion of thyroglobulin, previously discussed. The third step involves I^- oxidation by the thyroid peroxidase (TPO) enzyme, in which the iodide is moved to the follicular lumen to be oxidized to iodine, followed by the organification of the thyroglobulin, which includes iodination of the tyrosine residues to form MIT and DIT followed by T4 hormone synthesis [37,38].

Iodine released by deiodination of T4 can be reused for further thyroid hormone synthesis or it can be excreted mainly through urine. In humans, approximately 90% of the I^- is excreted through the kidneys. Iodine excretion has been correlated with iodide intake, a small amount of iodide has been reported to be excreted through feces and sweat [38]. In horses, renal excretion is highly associated to I^- intake, indeed, measurement of creatinine:iodine ratio has been reported to be a sensitive method to assess I^- intake, whereas, fecal excretion has not been associated with iodide intake in horses [35].

2.4.2 Iodine metabolism in the foal

Iodide metabolism in the foal is believed to be similar compared to the adult horse. Nevertheless, renal maturation in the neonatal foal is not fully accomplished until at least 4 weeks after birth. Regardless of having the maximum number of nephrons before birth, there is still a significant increase in glomerular volume during the first months after foaling, suggesting that adequate glomerular filtration is not completely achieved in the neonatal period, as seen in the sheep and rat [39]. Thus, the equine neonate has physiologic similarities to what has been reported in neonatal calves, where there is a lack of an efficient excretion route for iodine, predisposing to thyroid dysfunction due to iodine excess [40].

2.4.3 Reference range in equine serum and milk

Serum iodine references ranges in adult horses are based on Puls [41] and are as follows: Deficient = < 10 µg/L; Marginal = 11 - 20 µg/L; Adequate = 20 - 49 µg/L and High = > 50 µg/L. Milk iodine reference ranges in mares based on Puls [41] are: Deficient: < 4 µg/L; Adequate: 10 - 16 µg/L; and Toxic: 50 µg/L.

2.4.4 Iodide requirements in mares and foals

There is a lack of robust information on I^- requirements in adult horses and foals, and some values have been adapted from other species. An intake of 3 – 5 µg/kg body weight (BW) I^- per day for adult horses is required for maintenance, and during exercise 5 µg/kg /BW I^- per day, with a maximum intake of 10 µg/kg /BW I^- for broodmares and foals has been recommended [35]. It is worth mentioning that iodine requirement in the last trimester of pregnancy is higher due to the exponential growth and maturation that the fetus undergoes.

2.4.5 Comparative Iodine levels in serum and milk among species

Iodine levels have been reported in mare's milk to range from 4 – 450 µg/L in healthy mares [41–43]. Iodine levels in milk were reported to decline by month of lactation post-partum [42].

Silva et al (1987) reported milk levels from mares that had been fed an excessive quantity of iodide in pregnancy (350 mg daily) that resulted in abortion, fetal and neonatal goitres. The mares fed the excess iodine had milk iodine levels that averaged 473 µg/L and had values ranging from 175 - 1,380 µg/L, and a group of healthy mares were reported to have average milk iodine levels of 124 µg/L ranging from 30 – 450 µg/L [42].

Mochizuki reported serum iodine ranging from 17.5 – 29.3 µg/L. Serum iodine in the mares fed a seaweed supplement containing excessive amounts of iodine averaged 338 µg/L (range 20 – 70) and healthy mares averaged 39 µg/L (range 30 – 57.5) [8,44].

Table 2.3. Serum reference ranges (µg/L) in serum, colostrum and milk samples among other domestic species. Literature references for these iodine values are shown in square brackets.

Species	I serum µg/L	I colostrum µg/L	I milk µg/L
Horse	20 – 49 [41]	unknown	4 – 124 [41–43,45]
Donkey	12.9 – 38.7 [46,47]	unknown	7, 12.8 - 231.9 [46,47]
Cow	99 – 175 [48,49]	87[48]	100 – 900 [43,47,49]
Goat	5.4 – 36.6 [49]	unknown	60 - 130, 575 [49]
Sheep	27, 60-100 [50,51]	57, 2083 [50]	1423 [49], 192 [43,47,50]
Human	65-164 [52]	21 –304 [36]	62l [53]

2.5 Thyroid function and iodine during pregnancy and peripartum period

2.5.1 Role of placenta in thyroid hormone delivery to the fetus

To the author's knowledge there is very little information regarding thyroid hormone delivery to the equine fetus during pregnancy in the mare. Allen and coworkers (1995) evaluated THs levels from equine fetuses collected at an abattoir. They showed no detectable T4 and T3 were present until the 4 month of pregnancy, when T4 was measured at 47 nmol/L, then a steady parallel increase in T4 and T3 levels occurred until the 9th month of gestation with T4 measuring

a mean of 353 nmol/L and T3 2.6 nmol/L, after which levels plateaued in late pregnancy [54]. More research has been conducted in humans and sheep on the role of placenta in thyroid hormone delivery to the fetus. It is well documented that placental transfer of thyroid hormones from maternal to fetal circulation is quite variable among domestic species, and this difference relies on the type of placentation. The hemochorial placenta found in humans, dogs and cats are reported to be permeable to maternal THs through the whole pregnancy, whereas the epitheliochorial placenta found in horses, bovine and small ruminants have been found to be impermeable to THs at least from the second half of pregnancy [11]. In addition, it has been proposed that thyroid hormone delivery to the fetus before the complete maturation of the fetal HPT axis in species with epitheliochorial placenta, approximately before the 5^{th} or 6^{th} week of gestation in the sheep, is through maternal transfer of TRH to the fetus as the placenta can be permeable to TRH [21]. The placenta not only regulates the delivery of thyroid hormones from the dam to the fetus, but along with fetal liver, kidneys and thyroid gland it also maintains adequate levels of THs in fetal circulation at every stage of gestation.

Factors such as iodothyronine deiodinases, sulfotransferases, sulfatases for metabolism of THs, thyroid hormone transporters and thyroid hormone binding to proteins in the trophoblast cells have been reported to modulate the process, in which only the expression of D3 and sulfation of THs have been demonstrated in domestic animals [55]. In human and ovine placenta, D3 is reported to be highly active in the placenta, it metabolizes the majority of maternal T4 to the inactive rT3 and limits its exposure to the fetus, also it has been suggested that D3 contributes to the release of iodine into fetal circulation for fetal thyroid hormone synthesis [11,55], for that reason, high levels of T4 and rT3 along with low levels of T3 compared to the postnatal life are reported in both human and ovine fetuses. Furthermore, it has been reported that approximately 80% of the T4 is metabolized to biologically inactive sulfated forms through sulfotransferases in fetal liver, kidneys and brain. It is worth mentioning that sulfated forms can be converted back to T3 by sulfatase enzymes in liver, lung, brain and placenta, suggesting an efficient mechanism against cases of hypothyroidism, in which a constant supply of adequate levels of T3 is important for normal brain development during pregnancy [11].

2.5.2 Role of placenta in iodine delivery to the fetus

There are no direct studies performed in the horse on iodine uptake by the fetus; however, it has been demonstrated in humans and postulated in all mammalian species that the placenta plays a key role in the active transport of iodide from maternal to fetal circulation, which despite the type of placentation maternal iodide delivery to the fetus is essential for normal fetal thyroid hormone synthesis [11]. Diffusion of thyroid hormone may occur in early to mid – pregnancy [54]. In women, the presence of iodide transporters, a NIS, similar to what has seen in the mammary gland and thyroid gland, in the syncytiotrophoblastic cells of the human placenta have been demonstrated. In addition, the placenta is reported to have higher iodine levels compared to other tissues, suggesting the ability of the placenta to store iodide. The comparison of iodine content in placentas from women giving birth at term and based on iodine intake was performed to determine the role of the placenta on iodine storage and whether the delivery of iodide to fetal circulation is related to iodine content in the diet [56]. The author showed that iodine content in the placenta is associated with dietary iodine intake, which confirms the efficiency of the placenta to store iodide at least in the last trimester of pregnancy [56]. Due to the ability of the placenta to concentrate iodide and that can be affected by iodine intake, hypothyroidism during fetal development can be achieved from iodine excess due to a failure of maternal iodine excretion through urine and high amount of iodine exposure *in utero*.

2.5.3 Fetal growth and maturation

Maternal THs can have an indirect effect on fetal growth by altering placental development. Silva et al. (2018) stated that maternal THs are directly involved in proliferation, differentiation and endocrine functions of the trophoblastic cells due to high binging of THs to receptors present in the syncytiotrophoblast cells of the human placenta[21]. It has been documented that maternal thyroid dysfunction in the pregnant women can lead to preterm delivery, abortion, mental deficits in the newborn and growth retardation [21]. In addition, hypothyroidism can impair fetal weight at birth by affecting the vascular development and increasing the apoptotic rate of the placenta in rats, suggesting that alterations in placental vascular development can be present in hypothyroid women with spontaneous abortion and pre-eclampsia, and adequate THs concentrations play an important role in normal placental development [57]. The same effect has

not been proven in horses, however, Wilsher et al. (2013) examined the placenta of eight Thoroughbred foals born at term with moderate to severe degrees of congenital flexural deformities[58]. The most common finding was a notable reduction in the allantochorion dimensions mostly in the body and pregnant horn, with long avillous bands on the chorionic surface at the base of the pregnant horn [58]. It is worth noting that six out of the eight foals had a history of dystocia, and four were euthanized due to the severity of the flexural deformities. More studies need to be performed to determine if there is a direct association between iodine deficiency and inadequate fetal exposure to maternal THs during the critical period of placenta and fetal development in the horse, which can contribute to congenital flexural deformities.

Furthermore, adequate levels of maternal and/or fetal THs are essential for normal fetal growth in all mammalian species. The most remarkable example on the importance of regulatory effects of THs on fetal growth is reported in species with little placental transfer of maternal THs to fetal bloodstream, such as small ruminants and horses, in cases of fetal thyroid hormone deficiency. Allen et al (1998) reported the negative impact on intrauterine growth followed by partial fetal thyroidectomy at 215 days of gestation. The most noticeable changes were marked thyroid hyperplasia, signs of immaturity, severe tendon laxity, depression and lethargy, and inadequate carpal and tarsal bone ossification in two foals [59], suggesting that fetal thyroid hormones are necessary for fetal mass and cell differentiation of the musculoskeletal system during the second and last trimester of pregnancy in the horse. During skeletal development *in utero*, TSH acts directly in cartilage and bone formation by enhancing the expression of TSH receptor in chondrocytes, osteoblasts and osteoclasts. Similarly, chondrocyte differentiation and vascular invasion of cartilage is reported to be highly sensitive to T3, indicating that thyroid hormones are critical for endochondral ossification, control of chondrocyte maturation, cartilage matrix synthesis and linear growth [20]. Deiodinases type 2 activity have been reported to be essential for osteoblast function, maintenance of bone structure and strength. For that reason, it is not uncommon to find skeletal dysplasia such as a broad face, scoliosis, absence of ossification centers and congenital hip dislocation in cases of congenital hypothyroidism in infants, similar to what is seen in congenital hypothyroidism syndrome in neonatal foals [20]. Depending on the stage of pregnancy at the time of thyroidectomy and duration of thyroid hormone deficiency in fetal sheep, reduction of bodyweight and skeletal growth of limbs and vertebrae are common features reported in affected neonatal sheep [11]. On the contrary, domestic animals with higher placental

permeability to maternal THs can diminish the negative effects of fetal THs deficiency by adequate maternal THs delivery to the fetus, which acts as a compensatory mechanism. Nevertheless, when maternal and fetal THs concentrations are reduced, severe neuromotor, auditory, skeletal and respiratory abnormalities can arise [11].

Endocrinologically, premature infants are usually low in serum T4 and T3 concentrations along with immaturity of several tissues and physiological systems, similar to what has seen in premature foals and lambs. In sheep, normal endocrinologic changes near term involves changes in thyroid hormone metabolism by enhancing T3 release and reduce its clearance. This process is achieved mainly by increasing hepatic D1 activity, which increases T4 deiodination to T3, and reduces the expression of D3. The rise of T3 close to term is well documented in several domestic species and is essential for final maturation, synthesis and release of the surfactant by type II pneumocytes, of cardiomyocytes by increasing its size and population along with ensuring activation of physiological processes essential for neonatal survival such as thermogenesis, hepatic glucogenesis and pulmonary exchange [11,23]. In addition, cortisol acts as an important regulator of several iodothyronine deiodinases during late gestation, thus, the lack of endogenous prepartum surge of cortisol decreases the up regulation of renal and hepatic D1 activity with no down regulation of D3 activity in placenta, preventing the prepartum rise of T3 [23].

Ten out of fourteen neonatal foals born with moderate to severe musculoskeletal abnormalities had low TT4 and TT3 concentrations at birth with thyroid hyperplasia in six of them, confirming the negative impact of THs deficiency during the final growth and maturation of the musculoskeletal system in the equine [60].

2.5.4 Thyroid hormones and iodine in the neonatal period

Cellular and metabolic changes induced by thyroid hormones have a direct impact on cardiac, liver and respiratory systems during the transition to extrauterine life. Activation of hepatic gluconeogenesis at birth are mediated by THs. The ability to maintain adequate levels of glucose between placental separation and onset of suckling are dependent on normal glycogen storage and glucogenic enzyme activities in the liver, which is activated by the prepartum rise of cortisol and T3 [11]. Without glycogen storage, the neonatal foal will not be able to stand and

ingest colostrum. In addition, all mammalian neonatal species require an increase in heat production compared to *in utero*. Activation of the non-shivering thermogenesis at birth are dependent on THs, thus, the inability to maintain adequate body temperature can prevent oxygen consumption by the liver, brain, and adipose tissues. Furthermore, gas exchange and lung ventilation in the neonatal period depend on removal of lung liquid and surfactant production, and thyroid hormone concentrations play a key role in the process after birth [11].

Iodine levels in the neonatal period in horses have not been fully determined; however, it can be postulated that due to the dramatic increase in metabolic demand that leads to an increase in THs synthesis and release, iodine levels will also be expected to be high in the neonatal period

2.5.5 Methods for determination of iodine levels

Iodine is measured using a variety of laboratory methods including: capillary electrophoresis, ion chromatography, high-performance liquid chromatography, gas chromatography, spectrophotometry, ion-selective electrode, polarography, voltammetry, atomic emission spectrometry, and neutron activation analysis. Measurement of iodine in serum, urine and milk is accomplished mainly by two methods. The kinetic spectrophotometric method is called "Sandell-Kolthoff reaction". It has been successfully applied to determine protein bound and total inorganic serum iodine, urinary iodide, food, feces and amniotic fluid. However, high concentrations of thiocyanate, some trace metal ions like silver or mercury, and nitrite, ascorbic acid and iron interfere with the reaction, which decreases its sensitivity in cases when exposure to nitrites or GSL has occurred [61,62]. Another method is inductively coupled mass spectrometry (ICP-MS) which is a highly sensitive method. This occurs by ionizing the sample with coupled plasma followed by separation and quantification of the ions by a mass spectrometer, even though it is not the best test to measure iodine, the sensitivity is higher compared to the rest of the methods [61]. Iodine content in urine and milk are commonly analyzed worldwide for nutritional and epidemiological studies in humans. A modified protocol based on alkaline ashing prior to final determination for iodine by the Sandell and Kolthoff method increased the sensitivity of this test by removing the organic matter that can interfere with the result [43]. Iodine is also quantified indirectly by measurement of thyroid hormones as the thyroid hormones are the main organic form of iodine [18].

2.6 Nutritional factors influencing thyroid function

Important nutritional factors associated with thyroid function includes minerals and vitamins such as iodine, selenium, zinc, copper iron and vitamin A; exposure to plants containing goitrogens such as certain GSL in *Brassica spp.* family plants or potentially fescue grass infected with fungal endophytes (*Neotyphodium coenophialum*), and chemical compounds such as nitrates, nitriles and perchlorates which can interfere with iodine uptake, thus causing thyroid dysfunction [63–66].

2.6.1 Type of feed (winter vs summer)

Horses are non-ruminant grazing animals adapted to life in a prairie grassland ecosystem. Many horses are stabled throughout the year and may only be fed prepared feeds such as hay and concentrate. Winter grazing is typically limited, and most horses are fed or supplemented with forage. When provided access to pasture, horses have been observed to graze for 10 - 15 hours per day in 10 - 15 feed-bouts [64,67]. Current recommendations include performing a complete nutrient analysis with an estimate of the energy content of the hay and concentrates. The quality of prepared feed is quite variable and having hay free of excess dust, mold and spoilage is important for equine health. Horses have a minimum requirement for forage and it is currently recommended that 12.5 g DM per kilogram body weight is fed to horses to meet both behavioural and caloric needs[35]. Many horse owners feed concentrates to their horses as they believe the energy requirements of the horses are too high to be met by grazing [64]. Concentrates and forages in Western Canada are typically very low in important trace minerals such as iodine, copper, zinc and selenium [68,69]. North America has many regions with iodine deficient soil, including the prairies, and this has historically resulted in goiters in animals and peoples [70,71]. Trace minerals are important for thyroid function. Iodine is required to synthesize thyroid hormone, and zinc, copper and selenium are required for general metabolism [65]. Additionally the tissue deiodinases that convert T4 to T3 are selenoproteins, and selenium is a component of glutathione peroxidase [72]. Soils and feeds in Saskatchewan are often low in these four nutrients, and this may result in mineral deficiencies [68]. Soil zone is an important determinant of copper status in cattle [73].

While there is little information on horses, copper and vitamin A levels were noted to be higher after summer grazing, while molybdenum and selenium were lower in beef cows [74] . Lush green pasture is high in beta carotene and tochopherols and tochotrienols (vitamin E). Pasture

has been shown to decrease in nutritive value over time as the plants mature [64]. Mature forage has limited amounts of these vitamins [68]. Pastures across western Canada are known to contain a large variety of weeds, most of which are not native to North America [75]. Heat maps of where the weeds are found are available. Many of these weeds contain nitrates or GSL [75]. Additionally, volunteer plants from adjacent oil seed crops are also present in some locations.

2.6.2 Type of mineral supplements

Horse owners have access to a variety of feed products and may provide minerals in the form of blocks, loose salt or mineral mixtures, and ration balancers. Voluntary intake of mineral supplements is variable and ranges from 30 – 60 g per day [68]. Salt consumption may range from 10 – 100 g per day. Lactating mares and working horses may consume more salt, and will consume more loose salt than salt from a block [68,76]. Due to various regional differences different types of mineral blocks or mineral mixtures may be recommended [77]. Salt blocks were developed for cattle and may not be the optimum product for mineral supplementation of horses. It is common to mix mineral and iodized salt to increase intake in horses [68], others may choose to top dress minerals onto a concentrate ration. As Saskatchewan forage and water sources may contain high levels of alkaline salts, livestock may not crave salt, which is used as the main means of providing iodine [68].

2.6.3 Commercial supplements

Table 2.4 shows trace mineral content of a number of equine supplements.

Table 2.4. Select trace mineral content of a number of commercial supplements commonly fed to horses. *Sea salt, EDDI: Ethylenediamine dihydroiodide*

Product	Selenium	Copper	Zinc	Iodine	Form
Weatherguard 22	10 mg/kg Sodium selenite	4000 mg/kg Copper sulfate	8000 mg/kg Zinc oxide	50 mg/kg Calcium iodate	Loose mineral

Platinum Performance	6 mg/kg Selenium yeast	106 mg/kg Copper gluconate	909 mg/kg Zinc gluconate	3.78 mg/kg Iodine chelate	Feed in scoops 66 g/each
Platinum CJ	5.12 mg/kg Selenium yeast	83.3 mg/kg Copper gluconate	768 mg/kg Zinc gluconate	3.2 mg/kg Iodine chelate	Feed in scoops 78g/each
Platinum GI	5.44 mg/kg Selenium yeast	95 mg/kg Copper gluconate	816 mg/kg Zinc gluconate	3.4 mg/kg Iodine chelate	Feed in scoops 73.5g/ each
Greenline E plus selenium	30 mg/kg Sodium selenite	0	0	0	Feed in scoops
Strictly equine E + Se	70 mg/kg selplex	0	0	0	Feed in scoops
Farnum Mare Plus	0.18 mg/kg Sodium selenite	318 mg/kg Copper amino acid chelate	909 mg/kg Zinc amino acid chelate	73 mg/kg EDDI	Feed in scoops corn based
Morton iOFIXT	0	350-450 mg/kg Copper oxide	3500 mg/kg Zinc oxide	70 mg/kg Pentacalcium orthoperiodate	Loose mineral
Redmond Selenium 30	30 mg/kg Sodium selenite	300 mg/kg Copper sulphate	3500 mg/kg Zinc oxide Zinc gluconate	110 mg/kg EDDI	Loose mineral
Redmond Selenium 90	90 mg/kg Sodium selenite	300 mg/kg Copper sulphate	3500 mg/kg Zinc oxide Zinc gluconate	110 mg/kg EDDI	Loose mineral
Saltec Iodized salt for livestock	0	0	0	150 mg/kg EDDI	Loose mineral

2.6.4 Organic vs inorganic iodine

There are a variety of ways of supplementing iodine (I_2) to livestock in feeds. Most involve the addition of inorganic iodine compounds. Iodide (I^-) and iodate (IO_3) feed additives are common such as: anhydrous calcium iodate [$Ca(IO_3)$], potassium iodide (KI), potassium iodate (KIO_3), cuprous iodide (CuI), sodium iodide (NaI), sodium iodate ($NaIO_3$), sodium periodate ($NaIO_4$) or organic iodide such as casein bound iodine supplements, or ethylene diamine dihydroiodide (EDDI) [78,79]. The EDDI is reported to have high bioavailability and iodate supplements are

reported to be very stable. In tropical environments potassium iodide and potassium iodate are preferred because of their stability (WHO 2007). Iodate is reduced during the process of digestion and is absorbed as iodide [78].

There have been a number of feed trials that show no major difference in livestock performance when similar amounts of different iodine sources are utilized within the recommended ranges [81]. The retention of iodine may be affected by the type of iodide fed [82]. Milk iodine is determined largely from the level of iodine in the feed, although in dairy cows a significant level of iodine in milk is derived from iodine based teat dips and sanitizer [83,84]. Fortification of animal feed with iodine has been one means of delivering iodine to human populations, as higher levels of iodine supplementation in feed has resulted in higher iodine levels in milk and egg yolks [79]. While regulations concerning mandatory salt iodization have prevented a large number of iodine deficiency disorders, iodine deficiency still impacts 1.6 billion people worldwide [85]. Important source of dietary iodine include: seafood, fish, seaweed, and cow's milk which is one of the most common sources of iodine for people in many countries [79].

2.6.5 Microminerals and substances that are goitrogenic or are antagonistic to iodide uptake

A variety of other compounds or minerals called iodine antagonists affect the absorption of iodide or they disrupt the function of the thyroid. Mechanisms that may be impacted include changes in or disruption of: iodine (I_2) transport, thyroid peroxidase level/activity, thyroid hormone-binding proteins, hepatic catabolism, deiodinases, and receptor binding [86]. Many animals that exhibit one trace mineral deficiency on close examination will be found to have deficiencies in other minerals. Common trace mineral deficiencies include: iodine, selenium, copper and zinc; however in humans vitamin A and folate may also be lacking and are required for thyroid health [65,68,87]. The effects of various minerals on thyroid function has been reviewed by Sarne 2016 [87].

Calcium. Calcium has been reported to be goitrogenic when fed in excess. Administration of 2 g calcium per day decreased iodide clearance by the thyroid and reduced the absorption of T4 [87]. This may be somewhat relevant to horses as many are fed various supplements and provided free

access to round bales of alfalfa which are known to contain high amounts of calcium, which may lead to a dietary imbalance in calcium and phosphorus [68].

Bromine. Bromine is a halide element that occurs naturally in the environment, but environmental levels in some areas have been increased by use of bromine in pesticide sprays and due to pollution from industrial processes. Bromine is concentrated by the thyroid gland and interferes with the uptake of iodine in animals and people, probably by competitive inhibition of iodide transport into the thyroid follicular cells. Bromine concentrations in high amounts in animal feed produces disease, lowers thyroid iodine concentrations, and causes liver damage [88]. It is worth mentioning that Bromine has been identified in the formation water associated with Potash mines in the province of Saskatchewan [89].

Rubidium. Rubidium is an alkali metal which is goitrogenic in rats and accumulates in abnormal thyroid tissue; however, the mechanism of action is unknown [87,90]. Rubidium is a highly reactive element and oxidizes when in contact with water. It is used in nuclear imaging.

Fluorine. Fluorine is an element that is not concentrated by the thyroid. In high concentrations it has been shown to be goitrogenic in animals, but levels associated with endemic fluorosis are not sufficient to interfere with thyroid function or to produce goiter. Iodine deficiency may be exacerbated by high dietary fluorine, and contribute to goiter formation [87] . There are two mechanisms by which fluorine causes thyroid dysfunction; inhibition of the Na+, K+ ATP ase activity and NIS gene expression, thus, decreasing iodine uptake not only by the thyroid gland but by gastric mucosa and mammary gland tissue [91]. Fluoride poisoning in endemic areas have been reported in various domestic animals, including the horse, with dental and severe skeletal fluorosis. The clinical signs include intermittent lameness, hoof deformities, and periosteal exostosis [92].

Cobalt. Cobalt has been reported to inhibit iodine binding by the thyroid. Cobalt deficiency causes a reduction in type I monodeiodinase activity and a decrease in T3, while cobalt excess may produce goiter and decreased thyroid hormone production, and has been used to treat thyrotoxicosis [87]. Cobalt and iodine containing salt blocks are a common mineral supplement for horses [68,77,87].

Cadmium. Administration of cadmium has been reported to have variable effect in humans where thyroid hormones increase and in rats or mice and serum levels of T4 and T3 decrease [86]. Cadmium also decreases the activity of hepatic Type I - 5'Deiodinase [87].

Lithium. Lithium has been utilized in the treatment of manic-depressive psychosis in humans. Lithium administration increases thyroid weight and slows thyroid iodine release. Lithium is concentrated by the thyroid, it inhibits the adenylate cyclase activity, blocks the cAMP-mediated translocation of thyroid hormone and stabilizes thyroid microtubules, hence there is a corresponding increase in the intrathyroidal iodide concentration. Hypothyroidism results in up to 40% of patients treated with lithium, and the prevalence of goiter has been reported to be as high as 60%, and lithium therapy has been infrequently associated with the development of thyrotoxicosis [87].

Selenium. The thyroid contains high levels of selenium. Selenium is integral to thyroid function because it is contained in glutathione peroxidase (GSH-Px) and superoxide dismutase. Additionally, deiodinase enzymes contain selenium and are sometimes referred to as selenoenzymes. Most references that show positive relationships between iodine and selenium are from animal studies [93]. Selenium deficiency could exacerbate iodine deficiency by impairing the ability to regulate oxidation and may lead to decreased peripheral conversion of T4 to T3 [94] . Treatment of goitrous children with selenium and iodine deficiency leads to a reduction in serum TSH and goiter size. The response was correlated with the baseline selenium level. In an epidemiological study in China, low selenium levels were associated with an increased incidence of goiter, sub-clinical and overt hypothyroidism and thyroiditis [87].

Zinc. Zinc is one of the micronutrients commonly deficient in humans and livestock [68]. The level of zinc is positively correlated with iodine [65]. Thyroid hormone binding transcription factors contain zinc, but whether they are affected by deficiency is unknown [87].

Iron. Adequate iron is needed for good general health. The combination of iodine and iron deficiency contributes to parasitosis [95]. Iron level is also positively associated with iodine [65].

Copper. Dietary copper utilization is sensitive to inhibition by other minerals that may be concurrently consumed including iron, molybdenum, and sulfur. These elements may influence

the bioavailability, absorption and accumulation of copper [94]. Copper deficiency results in changes in selenoglutathione peroxidase activity and selenodeiodinase enzyme activity in rats [87]. In horses copper deficiency causes changes in hair coat colour, limb deformities, immune system dysfunction, and connective tissue problems [96,97] .

Nitrates. A concentration of 0.3 - 0.9% nitrate in the diet can interfere with ^{131}I uptake by affecting the sodium iodine symporter in the thyroid. Significant concentrations of nitrate are found in pasture weeds and in some types of hay and silage [75,98]. Heavily fertilized pastures, weather stress on grasses in pastures, water contaminated with nitrate run off, green feed (immature cereal hay), and grazing in pastures with nitrogen containing weeds have been associated with hypothyroidism in horses and other livestock species [6,79,98]. Nitrate exposure has been linked to thyroid dysfunction in people [99].

Perchlorates. Perchlorates are substances that occur naturally in the environment but are also used in manufacturing processes and are contained in fireworks, munitions and are associated with environmental pollution. Perchlorate levels are often monitored in drinking water. Perchlorate is an anionic iodine uptake inhibitor. The mechanism of action is competitive inhibition of the sodium iodide symporter. Perchlorate exposure has also been associated with decreased free T3 and elevated TSH in humans. Higher exposure levels are likely of concern when iodine levels are low and demand for iodine is high such as during pregnancy. Perchlorate has been measured in human colostrum but was not correlated with colostrum and milk iodine level [36,53]. Nitrate and cyanide (SCN) levels are reported to be more important as iodine uptake inhibitors than perchlorates in most settings [99]. Perchlorates have been identified in cow's milk and feed. Levels of perchlorate in milk were associated with a source of water used for the irrigation of alfalfa that was known to be contaminated with industrial pollution [100]. Few studies have been performed on perchlorates in domestic animals.

2.7 Thyroid function assessment in the horse

2.7.1 Basal hormone analysis

Basal TT3 and TT4 single point measurements are not considered to be reliable indicators of thyroid function in the horse, dogs, cats and humans. The majority of the circulating TT4 is bound to plasma proteins such as thyroxine binding globulin, serum albumin and transthyretin, and its production depends on metabolic rate and energy utilization, thus TT4 concentrations can be altered by a wide variety of medications and pathophysiologic states. Administration of phenylbutazone causes a decrease in TT4 levels by competing for binding carrier proteins, whereas, dexamethasone administration has been reported to decrease TT3 levels by inhibiting 5'-monodeiodinase. Similarly, high training levels, decreased appetite and food deprivation can alter THs circulating levels. Thus, it is recommended to avoid any type of medication for at least two weeks before assessing THs concentrations [19,101]. Likewise, TT3 levels have been reported to be within the normal range in canine hypothyroidism presumably due to compensatory mechanisms during the first stages of thyroid dysfunction [102]. It is worth mentioning that assessing fT4 by equilibrium dialysis or using an ultrafiltration method is reported to be more accurate if a single point measurement must be utilized, as free THs fractions are not directly influenced by the binding capacity to proteins. However, commercial diagnostic kits are not widely available for equine samples, are costly and it has been reported that measuring fT4 by direct serum analog assays results in a lower value compared to equilibrium dialysis [101].

2.7.2 Stimulation tests TSH, TRH

Measuring thyroid hormone concentrations and TSH levels by administering TSH or TRH to stimulate THs and TSH release is considered the most reliable indicator of thyroid function in the horse compared to single time point measurements. Nevertheless, the test requires blood samples at different time points, making it less efficient to perform in the field [19]. The TRH stimulation test consists of a single injection of TRH (1 mg - adult horse; 0.5 mg - foal) or TSH (5 IU) intravenously followed by blood sampling at 2 and 4 hrs post TRH injection. The T3 concentrations are expected to double at 2 hours post TRH injection compared to basal levels, whereas, T4 doubles at 4 hours post TRH injection in euthyroid horses. Breuhaus (2011) reported

that horses with high T4 and T3 resting values tend to fail to double their values after TRH, whereas, low resting values tend to have a higher response after the stimulation test, also, T4 and T3 peaks may happen either earlier or later than the time points previously mentioned in individual horses[19]. For that reason, a 1.3 – 3.8 fold increase over the basal TT4 level at 4 hours post-TRH administration or a 1.1 – 10.3 fold increase over the basal TRH TT3 levels at 2 hours post-TRH administration have also been reported to be related to an adequate response from the thyroid gland [19]. Meredith et al. (2004) reported a mean increase in T3 of 4.5 fold at 2 hrs post TRH, and showed no association with response to TRH and pregnancy rates [103]. It is worth mentioning caution should be taken when interpreting the results due to the inherent variability that is present in the test.

2.7.3 Ultrasound examination

There is some published information on the relationship between ultrasonographic dimensions of the thyroid gland and thyroid function in horses. The normal values for the dimensions of the equine thyroid gland measured by ultrasonography were reported by Davies et al. (2010) in a group of 10 horses, and described the use of an ellipsoid calculation that included the length, width and height of the gland [π /6 (length x width x height)] [14,15]. Viana et al. (2019) studied 11 horses and utilized (length x width x height) cm^3 to calculate thyroid volume [15]. Davies et al. (2010) reported a combined mean thyroid gland volume (cm^3) of 27 – 37 cm^3, and Viana et al. (2019) reported about 24 cm^3 [17,18]. The ultrasound evaluation is performed by applying mineral oil and/or alcohol in an area of approximately 6 x 6 cm caudal to the larynx on either side of the neck. An ultrasound machine with a 7.5 – 5 MHz linear probe with standardized power, and gain settings (G92, P100) can be used to examine both thyroid glands by displaying longitudinal images of each thyroid lobe to assess length and height (mm), and transverse images for width (mm) measurements using the internal callipers.

One of the goals of ultrasonographic assessment is to help determine whether the thyroid gland is within normal limits, enlarged, or smaller than average. Assessment of thyroid size is best performed by correcting for body weight or body surface area. Schotthauer (1931) reported a range of 0.02 – 0.06 g thyroid per kilogram of body weight as the normal range in a necropsy study. Enlargement of the thyroid gland may be due to goiter formation which is usually bilateral

[71]. Unilateral or bilateral enlargement may also be due to cystic and/or nodular structures that can be compatible with tumours or an increase in thyroid gland parenchyma [19]. In humans, ultrasound-guided fine needle aspiration biopsy (FNA) of the thyroid gland in relation to cytological features and ultrasonographic findings have been successfully used in the early diagnosis of thyroid cancer. Nodule size (>10 mm) along with ultrasound features such as marked hypoechogenicity, microcalcification areas, microlobulated nodules with/or irregular margins and taller rather than wide appearance have been reported to be associated with suspicious malignancy [15,104,105]. However, in horses there are no large studies performed in this area.

2.8 Thyroid dysfunction in the adult horse

Thyroid dysfunction can result from a decrease in the production of THs, a deficient thyroid activity or a disruption in HPT axis. Hypothyroidism is classified in three categories: primary hypothyroidism refers to the inhibition of thyroid hormone synthesis such as arises due to iodine excess or deficiency, thyroid neoplasia and goitrogenic compounds affecting the thyroid gland; secondary or central hypothyroidism refers to dysfunction at the pituitary or hypothalamic level; and tertiary hypothyroidism results in the failure of THs to exert their effects in peripheral tissues [10].

2.8.1 Primary hypothyroidism

The prevalence of primary hypothyroidism in adult horses is questionable. In dogs and humans, primary hypothyroidism is typically caused by autoimmune diseases and is commonly seen in clinical practice. Perillo et al. (2005) reported histologic changes, such as lymphocytic infiltration and fibrosis with progressive destruction of the thyroid gland, consistent with Hashimoto's thyroiditis like disease in 80% of the 156 thyroid glands recovered from 622 slaughtered horses in Eastern Europe [106]. Interestingly, the same group measured serum thyroglobulin levels in 9 horses with histologic changes similar to human Hashimoto thyroiditis, and reported levels to be significantly higher compared to euthyroid horses. However, it is unknown whether these animals displayed clinical signs compatible with thyroid dysfunction as

seen in dogs and humans. Similarly, a case of equine iatrogenic hypothyroidism in a 14-year-old gelding that was receiving 60 g/day of potassium iodide for 15 days was reported in Brazil (recommended daily intake is 0.00315 g). The most relevant clinical signs were decreased intestinal motility, hyperthermia, lethargy, goiter, reluctant to move and basal TT4 levels of 0.10 μg/dl [107]. There are a number of case reports from horses that received too little or too much dietary iodine which resulted in thyroid dysfunction [45,70,107–109]. There is one report of a herd of horses affected by primary hypothyroidism that was related to exposure to goitrogenic plants [110].

In addition, primary hypothyroidism has also been documented experimentally. Breuhaus (2002) reported THs and TSH serum levels after TRH stimulation test in euthyroid and induced-hypothyroid horses with the highly goitrogenic compound called propylthiouracil (PTU) in a 6-week period at a dose of 4 mg/kg daily [111]. Even though none of the horses showed clinical signs, basal TT3 and fT3 were markedly decreased after one week of PTU administration, whereas, TT4 was significantly decreased until the 4th week and TSH was dramatically increased until the 5th week of PTU treatment compared to euthyroid horses. In addition, THs failed to double after the TRH stimulation test, whereas TSH levels significantly increased 45 min after TRH injection due to the lack of negative feedback from adequate levels of circulating THs [111]. Propylthiouracil's affects THs synthesis as it inhibits iodination of the thyroglobulin tyrosine residues by blocking the TPO enzyme even if adequate iodine supplementation is present [112]. Other authors reported similar effects of PTU on thyroid function in horses using a similar protocol [33,113].

2.8.3 Nonthyroidal illness syndrome

Central or secondary hypothyroidism, also referred as nonthyroidal illness syndrome, is characterized by a lack of response of the HPT axis resulting in low THs levels, but also can result in a decrease in thyroid hormone binding proteins in bloodstream. Nonetheless, the thyroid gland itself is apparently normal [114]. Factors such as phenylbutazone and dexamethasone administration, high energy and protein diets, level of training, food deprivation and ingestion of fescue grass containing endophyte have been reported to decrease THs levels. Studies have shown that phenylbutazone at 4.4 mg/kg intravenously for five days decreases T4 circulating levels on

the last day of treatment and T4 remained low for approximately 10 days. Phenylbutazone has high protein binding and it displaces TH, thus, high fT4 levels remain high resulting in inhibition of the HPT axis. Similarly, low leptin levels caused by food deprivation or prolonged fasting for periods of time reduce the thyroid hormone negative feedback on TRH release [4,19].

On the contrary, nonthyroidal illness syndrome in neonatal foals is believed to be an adaptive response to systemic inflammation in order to prevent organ failure and death in critically ill foals[115]. Himler et al. (2012) discussed the mechanisms that are believed to cause low THs concentrations in this syndrome; in septic rats, an increase pro-inflammatory cytokines such as interleukin -6, tumor necrosis factor α, and interleukin -1β can inhibit 5'deiodinase activity, and an increase in iodine utilisation by neutrophils has also been observed to contribute to decrease THs concentrations [115].

2.8.4 Diagnosis and treatment

Diagnosis of hypothyroidism based on clinical signs alone is not reliable and as signs are inconsistent among horses. Thyroidectomized horses can present with clinical signs such as: hypothermia, coarse haircoat, limb edema, lethargy and abnormal growth rate in growing horses [113]. In addition, exercise intolerance, alterations in the cardiovascular system, and in some cases goiter have been reported [114]. Laboratory diagnosis includes low basal serum TT4 and TT3 concentrations, but because of baseline variability, are only supportive of the diagnosis of hypothyroidism in horses. It has been stated that horses should not be diagnosed as hypothyroid until it has been proven that the HPT axis is disrupted; however, the effects of various compounds and goitrogens on the HPT have not been thoroughly investigated in horses. As discussed above, several factors such as non-thyroidal illnesses and medications, can decrease THs levels without affecting the thyroid gland, thus, to differentiate primary hypothyroidism from other abnormalities in the HPT axis, TSH concentrations should be assessed. Below normal serum levels of free and total THs along with elevated TSH concentrations are indicative of primary hypothyroidism, whereas, low THs concentrations can result from other disturbances in the HPT axis [4]. The TSH assay is not commercially available for horses, and elevations in TSH are more commonly associated with autoimmune disease in animals such as dogs [116]. Furthermore, a TRH stimulation test is recommended to confirm abnormalities in the hypothalamic-pituitary thyroid

axis. Failure to double the basal value at 2 hr and 4 hr for T3 and T4, respectively, can suggest thyroid dysfunction; however, the test cannot confirm primary hypothyroidism unless TSH levels are assessed after TRH administration. Thyrotrophin levels should increase at 45 minutes after TRH administration, and should be more than double compared to basal resting values [4,19].

Ultrasonographic evaluation is recommended to determine if the enlargement of the thyroid gland is derived from neoplastic areas and or/cystic structures or if the gland itself is enlarged (goiter) [117]. Furthermore, a diagnosis of thyroid dysfunction based on necropsy is made mainly on the thyroid gland weight per kg body weight, and histological findings in goitre include; hyperplastic thyroid gland epithelium, and small, irregular size follicles with minimal amount of colloid which has been reported in cases of adult thyroid disease, and in foals with congenital hypothyroidism syndrome [70,71,118].

Thyroid hormone supplementation, mainly thyroxine, has been prescribed to horses with suspected hypothyroidism and with other endocrinologic diseases, such as equine metabolic syndrome (EMS). Iodinated casein, which contains 1% T4 ("Thyroprotein") at a dose of 5 to 15 g per day orally and the synthetic thyroid hormone called levothyroxine sodium (L-T4), with doses ranging from 20 µg/kg BW per day to as high as 50 to 100 µg/kg BW per day orally are commercially available for horses [19]. Healthy mares receiving increasing doses of L-T4 starting at 24, 48, 72 and 96 mg per day for 8 weeks resulted in a median weight loss of 19 kg compared to 4 kg for the non-treated mares; TT4 basal concentrations were positively correlated with time, whereas TSH concentrations decreased by week 8 compared to week 0. In addition, TT4, TT3 and fT3 concentrations after a TRH stimulation test decreased significantly overtime with no significant effect on fT4 concentrations [119]. It is worth mentioning that heart rate (HR), respiratory rate (RR) and body temperature did not change among groups, however, changes in behaviour, such as being more excited and agitated, were observed only with 96 mg of L-T4, with no behavioural effects at dosages of 24, 48 and 72 mg [119]. Similarly, the effects of long-term oral administrations of L-T4 at a dose of 48 mg/day for 48 weeks on thyroid hormone concentrations and health status were assessed. Overall, basal TT4 concentrations increased overtime, whereas thyroid hormone responses to TRH stimulation decreased, there was enhanced body weight loss and there were no negative effects on general health in euthyroid mares [120]. It is worth mentioning that in both studies, the lack of TRH stimulation test response may have been

due to the suppression effects of exogenous L-T4 on the HPT axis. To the authors knowledge, there is a lack of information regarding the effect and pharmacokinetics of L-T4 on hypothyroid horses.

2.9 Thyroid dysfunction in foals

Low levels of THs have been associated with prematurity [121], but may also be related to severe illness in mature foals, as described by Breuhaus (2014), who compared resting T4 and T3 levels in 24-36 hrs old normal, sick and premature foals. Premature foals that died had a median (range) nmol/L TT3 and TT4 levels of 1.8 (0.4-7.4) and 127 (54-299), respectively, versus 4.3 nmol/L (1.3-15.9) for TT3 and 247 nmol/L (92-319) for TT4 in sick foals with a good survival prognosis, and 8.2 nmol/L (6 - 11.8) for TT3 and 260 nmol/L (191-318) for TT4 in healthy foals. As described above, despite the severity of the illness, sick foals with a mature HPA axis that experienced low TT3 levels resulting from the negative effects of inflammatory cytokines on T4 deiodination, had better survival rates compared to premature foals that had low TT4 circulating levels, suggesting that high mortality rates may be directly related to the inadequate maturation of the HPA axis, which may affect thyroid hormone responsiveness against the high metabolic demand in the neonatal period [24].

Furthermore, Panzani et al. (2012) demonstrated similar patterns of THs levels between spontaneous and induced foals in the first week of life, suggesting that if induction to parturition is performed when the fetal HPA axis is matured, there are no negative effects on neonatal survival and THs production. On the contrary, foals hospitalized for hypoxic ischemic encephalopathy and sepsis resulted in lower THs levels compared to spontaneous and induced healthy foals [122]. It has been suggested that hypoxia can activate the release of inflammatory cytokines such as tumor necrosis factor- alfa, which have been found in higher concentrations in the cerebrospinal fluid and serum from newborn infants experiencing hypoxic-ischemic encephalopathy, causing a decrease in THs production, similar to what has been reported in septic rats [115,123].

2.9.1 Goitre – iodine deficiency and excess

Congenital goiter is defined as an enlargement of the foal's thyroid gland along with low serum THs concentrations caused either by an iodine excess or deficiency in the mare's diet or

ingestion of toxins or goitrogenic plants during pregnancy [19]. In early stages of iodine deficiency, there is a transient increase in TSH secretion by the pituitary gland, which increases sodium iodide symporter activity to enhance iodide uptake by the thyroid gland along with a reduction in renal clearance and an increase in reutilization of iodine from THs degradation with no effect on circulating THs concentrations [124]. However, in moderate to severe iodine deficiency, TSH levels are maintained at a high level and T4 levels decrease with no change in T3 levels. In chronic cases, T3 levels are also low, and goiter will arise as a result of a lack of iodine for THs synthesis despite constant TSH release [124]. On the contrary, a compensatory effect after ingesting a large amount of iodide has been reported in rats since 1940. The acute Wolf-Chaikoff effect is characterized by a decrease in THs synthesis lasting 24 hours. It has been postulated that an abrupt increase in intrathyroidal iodine levels inhibits the thyroid peroxidase and intrathyroidal deiodinases activity, thus decreasing THs synthesis. However, in euthyroid people, there is an increase in renal iodine clearance and the sodium iodide symporter activity decreases its expression, thus, active transport of iodine into the thyroid gland is decreased to resume normal TH production [125]. Nevertheless, a fetus *in utero* or an adult with a previous history of thyroid dysfunction may fail to escape the Wolf-Chaikoff effect, which means that the thyroid gland is unable to activate the regulatory mechanisms that allow the prompt resumption of normal thyroid function, causing thyroid gland hyperplasia [125]. Despite an iodine excess or deficiency, the low THs circulating levels causes an increase in TSH release, the thyroid gland increases iodine uptake and T4 synthesis; however, there is a continued stimulation of the thyroid gland via TSH signaling as there is no negative feedback, which leads to hypertrophy and hyperplasia of the follicular epithelial cells, a lack of colloid and an increase in vascularity [118].

2.9.2 Congenital hypothyroidism dysmaturity syndrome

In the 1980s, a syndrome of thyroid gland hyperplasia and moderate to severe musculoskeletal abnormalities (TH-MSD) such as angular limb deformities, rupture of the common digital extensor tendon as a result of severe contracted tendons, mandibular prognathia and failure of carpal and tarsal bone ossification in neonatal foals was first described in western Canada [126]. Allen et al. (1994) reported the prevalence of the syndrome in a ten-year period. From the 2,946 equine fetuses and foals submitted to the Western College of Veterinary Medicine,

2.7% were affected by the syndrome and 2.5% of these cases had evident thyroid gland hyperplasia [126]. A case definition was developed [6,126]. In addition, the majority of the affected foals had prolonged gestation with a mean of 360 days with moderate to severe signs of immaturity and were hypothermic at birth. These foals had low basal levels of thyroid hormone and failed to respond to TSH [54,60]. A proportion of CHDS foals have adenohypophyseal hyperplasia of thyrotrophs [118,126,127]. For that reason, the term congenital hypothyroidism and dysmaturity syndrome (CHDS) in foals has been used to better exhibit the pathogenesis of the disease. Initially, CHDS was exclusively reported in western Canada, but it has also been reported in the pacific northwest region of the USA, eastern Canada and Europe [128]. Koikkalainen et al (2014) described the clinical and pathological features of CHDS cases reported in Europe [118]. The most remarkable clinical signs seen in all the cases were weakness and inability to stand, rupture of the common digital extensor tendon, incomplete carpal and tarsal bone ossification, mandibular prognathism, hypothermia and dysmaturity. It is worth mentioning that evident goiter was not reported in all cases; however, histologically, there was a marked hypertrophy and hyperplasia of the follicular epithelium of the thyroid gland in all cases, and all foals had low THs concentrations compared to normal values for neonatal foals, and enlarged pituitary glands which is consistent with primary hypothyroidism [118].

The exact aetiology of CHDS remains unknown, but risk factors such as exposure to nitrates and goitrogenic substances such as mustard plants, along with low trace mineral supplementation (mainly iodine and selenium) during pregnancy have been associated with the syndrome [118]. Western Canada is well known for the high production of GSL containing crops such as canola (*Brassica* family type), rapeseed, mustard seed as well as wild plants that can contain goitrogenic substances such as nitrates and GSL [75].

2.9.3 Diagnosis and treatment

Diagnosis of thyroid dysfunction in the neonatal foal is based on clinical and radiographic signs, histological findings and thyroid hormone levels. Due to the essential role of THs during normal organ development and growth, the clinical signs tend to be more significantly severe compared to an adult horse. In normal neonatal foals, THs concentrations are higher in the first week of life and slowly decrease to reach adult levels in the next month[25,122]. For that reason,

low THs levels and failure to respond to the TRH stimulation test along with clinical and radiographic signs previously discussed and thyroid gland hyperplasia confirmed by histology are considered diagnostic. Nevertheless, determining the cause is still challenging for the equine practitioner as there is a lack of robust information regarding normal milk and serum iodine reference ranges in neonatal foals that can help in the determining whether the cause was an excess or iodine deficiency.

Prevention of CHDS includes adequate mineral supplementation, mainly iodine and selenium, in areas with iodine and selenium deficient soils and avoiding heavily fertilized pastures and pastures with goitrogenic plants during pregnancy. Levothyroxine (T4) at a dose of 20 to 50 μg/kg/ day and T3 at 1 μg/kg/day orally has been administered to severely affected neonatal foals, however, the response is questionable [19]. Foals have extremely high thyroid hormone levels at birth that decrease over time which may be related to a reduced demand for iodine and hence foals if they survive become euthyroid over time [19,118].

2.10 Glucosinolates

2.10.1 Chemical structure and source

*Brassicaceae f*amily vegetables such *as Brassica oleracea* (cabbage, broccoli, cauliflower, kale and brussels sprouts) and *Brassicaceae* family plants including oil seed crops of *B. napus* (rapeseed meal) and *B. juncea* (mustard) contain bioactive metabolites stored in intracellular vacuoles in the root, seed, leave and stem, such as GSL, erucic acid and other sulphur compounds, that act as a defense mechanism against animals, insects, bacteria and fungi [129]. Nearly 200 different types of GSL have been identified and are composed of a sulfur bridge along with a β-D-thioglucose group, a sulphonated oxime moiety and a variable aglycone side chain composed of different amino acids [66]. They are classified into three main groups based on the amino acid precursor: aliphatic GSL (alanine, leucine, isoleucine, methionine), indole GSL (tryptophan) and aromatic GSL (phenylalanine or tyrosine) [130]. GSL composition and concentration are different among plant species, and highly depends on external stimuli such as climatic conditions, agronomic practices (harvest, fertilization) and stage of feed production. For example, short-term storage can increase GSL content, whereas long-term storage decreases them. Similarly, autumn

harvested crops have higher GSL levels compared to winter harvest, and it has been suggested that hot dry environments along with lack of water can increase the formation of sugars and amino acids, which are the precursors for GSL formation [66,129,131].

2.10.2 Metabolism

Glucosinolates themselves are biologically inactive, but secondary metabolites produced by their degradation through the glucosinolate-myrosinase system are known to exert biological effects, and the negative effects are correlated with the concentration and type of GSL present in the plant. Once the plant is mechanically damaged, GSL is released and hydrolyzed by a thioglucoside glucohydrolase enzyme called myrosinase, which is present in the plant and in the intestinal microflora [66,130]. Major GSL found in plants are sinigrin, progoitrin, glucobrassicin and glucoraphanin. Progoitrin or L-5-vinyl-2-thiooxazolidine is mainly found in rapeseed meal (*Brassica napus),* and it is hydrolyzed into goitrin, a potent goitrogenic metabolite that blocks iodination of tyrosine residues, thus, inhibiting T4 synthesis. Sinigrin is hydrolyzed into allyl isothiocyanate, whereas glucobrassicin and glucoraphanin's main metabolites are thiocyanates, isothiocyanates or nitriles. [130–132]. Isothiocyanates, thiocyanates and sinigrin are responsible for the bitter taste that reduces feed intake and are reported to interfere with iodine uptake by the thyroid gland, thus causing thyroid dysfunction.

2.10.3 Interaction of iodine with glucosinolates

Secondary GSL metabolites such as thiocyanates, isothiocyanates and allyl isothiocyanates can alter the iodine uptake by the thyroid gland through different pathways. It has been reported that they: increase iodine efflux by facilitating urine excretion, interfere with the thyroid peroxidase activity, inhibit the incorporation of iodine into thyroglobulin and inhibit the sodium iodine symporter, which not only decrease iodine uptake by the thyroid gland [66,133], but can also affect the uptake of iodine by the mammary gland [134,135]. Chandra et al. (2016) published information about the prevalence of goiter in 6 to 12-year old children located in areas with high iodine supplementation. The mean urinary iodine (UI) concentration was 231 μg/L (adequate range for children: 100 to 199 μg/L) suggesting that iodine intake was more than adequate among

the population; but not toxic as UI concentration ≥ 300 μg/L is considered toxic and is associated with thyroid dysfunction in humans. Despite adequate iodine intake and the prevalence of goiter was high in this population, mean urinary thiocyanate (0.857 ± 0.486 mg/dl) was significantly higher compared to non-endemic goiter areas (0.504 ± 0.197 mg/dl), and a positive correlation between urinary excretion of iodine and thiocyanate was identified, suggesting a relationship between goiter and thiocyanate consumption in this population. High urinary thiocyanate levels were associated with the consumption of bamboo shoot which contains progoitrin. It is worth mentioning that water hardness was significantly higher, ranging from 210 to 618 ppm due high levels of solids including salt and iodine. The authors reported that other factors can contribute to the high goiter prevalence in the area [133,136]

Similarly, the effect of thiocyanates on iodine levels in cow milk has been reported. Dairy cows exposed to rapeseed meal or rapeseed press cake with GSL concentration of 50-100 mmol/kg resulted in a decrease in iodine level in milk by one half to three quarters compared to cows receiving a rapeseed meal-free diet, suggesting that the sodium iodine symporter activity in the mammary gland is also affected by the presence of thiocyanates[83,135].

Franke et al. (2009) reported the effect of rapeseed meal (RSM) and two different types of iodine supplementation on blood, milk, urine and fecal samples in dairy cows. There was a significant increase in serum iodine, fecal and urinary iodine levels, whereas milk iodine levels were significantly decreased after exposure to RSM suggesting a reduction in iodine transfer to the mammary gland and thyroid gland. On the contrary, cows fed the diet without RSM with iodine supplementation had higher iodine excretion through milk, urine and feces [137].

2.10.4 Glucosinolates and thyroid function in horses and ruminants

Type and concentration of GSL are not the only factors associated with the severity of the adverse effects of GSL, but tolerance among animal species and age play a key role in the side effects. It has been reported that ruminants are less sensitive to feeds containing GSL compared to monogastric animals such as pigs, poultry and fish [66]. The microflora present in the gastrointestinal system of ruminants enhances transformation of GSL metabolites, thus decreasing their side effects. Nevertheless, chronic exposure and concentrations of 11 μmol/g GSL in the diet

induces iodine deficiency in dairy cows and inclusion of 31 μmol/g diet if GSL in the diet can result in thyroid dysfunction and decreased fertility. Laarveld et al. (1981) showed the goitrogenic effects of Midas RSM diets, at an inclusion rate of 19% resulting in a significant increase in TSH levels and lower TT4 levels after TRH stimulation tests in dairy cows [135]. In pregnant sheep, rapeseed meal containing 4.2 mmol of GSL for 382 days resulted in a significant decrease in TT4 and iodine levels in lambs from ewes continually exposed to the RSM diet, and lower iodine levels were measured in colostrum and milk [138].

In addition, Bobek et al. (1992) reported the effects of thiocyanate in various domestic animals in southern Poland. Cows exposed to *Brassica* plants commonly found in the area, where there is high incidence of goitre in humans and low levels of iodine in cow milk, resulted in higher concentrations of thiocyanate in blood and milk with no evident change in serum iodine levels [139]. It was also observed that increased levels of thiocyanate in blood are related to greater goitrogenic effects [139]. Similarly, Papas et al. (1979) reported the effects of feeding RSM containing low and high GSL concentrations on thyroid hormones, iodine and GSL levels on milk in dairy cows and calves. Cows fed the meal containing high GSL content resulted in reduced T4 levels, and histological changes in the thyroid gland of calves were evident [140]. Rats receiving milk from cows fed high GSL meal resulted in increased thyroid gland weights, which was prevented by iodine supplementation [140]. It is worth noting that in this study no intact GSL was detected in the milk of cows exposed to rapeseed meal, only inorganic thiocyanate was detected [140].

In horses, to the author's knowledge there are no studies performed on the effect of GSL on thyroid function in horses. Nevertheless, Cymbaluk (1990) and Oliviera et al. (2001) reported feeding different levels of canola meal in growing horses with no adverse effects in a 60 - 75 day period [141,142]. This suggests that feeding canola meal to horses GSL for short periods of time did not cause adverse effects on parameters such as weight gain. The cost benefit of adding various GSL containing ingredients to equine feeds has not been determined, as the tolerance to different GSL have not been fully described.

2.10.5 Role of glucosinolates in pregnancy

There is a lack of evidence regarding the presence of secondary GSL metabolites in the fetal fluids of pregnant domestic animals. Nevertheless, negative effects of GSL diets on pregnant animals have been widely reported. Maternal iodine transfer is essential for adequate fetal thyroid hormone synthesis, for that reason chronic exposure to GSL during pregnancy can decrease iodine uptake by the maternal and fetal thyroid gland and increase urine excretion, thus causing thyroid dysfunction in the fetus. Pregnant sows exposed to chronic rapeseed meal diet without iodine supplementation resulted in prolonged gestation and stillborn piglets with enlarged thyroid gland and undetectable serum TT4 concentrations. Piglets from sows receiving rapeseed meal without iodine supplementation had significantly lower TT4 levels in serum compared to piglets from sows receiving iodine supplementation; however, in both sows and piglets iodine serum levels were higher compared to control group, suggesting that the lack of iodine transport to maternal and thyroid gland may cause an increase in serum iodine levels [143]. Schöne and Rajendrum (2009) have published a review on iodine in farm animals that summarizes many studies [79].

Ewes receiving 4.2 mmol of GSL and 4 g of nitrate without iodine supplementation resulted in congenital goiter and low iodine and TT4 serum concentrations in the respective lambs, whereas colostrum and milk samples had significantly lower iodine levels compared to the control group and ewes receiving iodine supplementation [138].

2.10.6 Methods for determination of glucosinolates

Glucosinolates are chemically stable compounds. A variety of methods have been utilized to measure GSL including indirect analyses based on the degradation products of GSL. Some assays evaluate the glucose content in the sample, such as the thymol test, which provides an estimate of the total amount of GSL. Sinigrin and progoitrin, which are GSL degradation products, have been analyzed using ELISA. Indole GSL levels may be detected after reaction with diazotized sulfanilic acid and isothiocyanate may be detected by its reaction to 1,2 benzendithiol using spectrophotometric methods. Near infrared spectroscopy of samples has been utilized to detect GSL but various other chromatographic methods such as ICP-MS, high performance liquid chromatography, and gas chromatography are more commonly applied [144]. Thiocyanate

concentrations in feed, serum, urine and milk has also been determined using ion chromatography tandem mass spectrometry [145,146]. There is no information regarding GSL levels in serum, urine and milk from horses.

For example, a determination of isothiocyanates in human plasma after consumption of raw broccoli has been reported. Blood samples were taken with EDTA tubes 3 hours after ingestion of broccoli, based on previous research in which the peak of isothiocyanates in blood was seen at the time previously mentioned. Initially, the plasma was treated with 100 mM acetic acid in methanol, was left on ice for 30 min and was then centrifuged. The supernatant was analysed using the liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) for GSL content. It is worth mentioning that this method has been used to analyse feed and other biologic samples [145].

CHAPTER 3

Effects of the Glucosinolate Sinigrin in combination with a Non-Iodine Supplemented Diet on Serum Iodine and Thyroid Hormone Concentrations in Non-pregnant Mares

TRANSITION PAGE

Authors: M.F. Lopez-Rodriguez[a], N.F. Cymbaluk[a], T. Epp[a], B. Laarveld[b], E.C. Serrano Recalde[a1], E. Simko[c], and C.E. Card[a]

Institutional affiliations: [a]Dept. Large Animal Clinical Sciences, [b] Dept. of Animal and Poultry Science, [c]Dept of Vet. Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4, São Paulo University, R. Araraquara – Vila Tabajara, Pres. Prudente, São Paulo, Brazil

Source of funding: Funded by the Mark and Pat DuMont Equine Orthopedics Research Fund

This chapter concerns the results of a three-month clinical nutritional trial designed to evaluate the combined effects of the glucosinolate sinigrin (GSL) and a low iodine diet on serum iodine and thyroid hormone concentrations in non-pregnant mares. Normal thyroid function is not only essential in the non-pregnant mare, but also plays a key role in the development and maturation of the equine foetus during pregnancy. The GSL used in this trial has been reported to affect iodine uptake by the thyroid gland in other farm animals. However, this is the first study to evaluate the effects of GSL in horses along with a low iodine supplemented diet, which are risk factors that have been postulated to cause congenital hypothyroidism dysmaturity syndrome in foals. In addition, the chapter critically evaluates thyroid function through Thyrotropin Releasing Hormone (TRH) stimulation tests and serum iodine measurement at trial initiation and week 12, rather than simply evaluating basal concentrations of these hormones.

3.1 Abstract

Exposure to plants containing glucosinolates (GSL) affects thyroid function in many species, and in horses is implicated in the birth of foals with congenital hypothyroidism. The present study was performed to determine the effect of feeding a GSL (sinigrin) in combination with low iodine diet for 12 weeks on thyroid hormones and serum iodine concentrations in non-pregnant mares. Nineteen mares aged 2 to 14 years were divided into Control (n=6), Low GSL (20 mmol/day) (n=7) and High GSL (35 mmol/day) (n=6) groups. Thyrotropin releasing hormone (TRH) stimulation tests and serum iodine measurements were performed at 0 and 12 weeks. Total triiodothyronine (TT3), total thyroxine (TT4) and thyroid stimulating hormone (TSH) concentrations were compared at baseline and post-TRH samples. The difference in concentrations (Δ) and fold change (FC) before and after TRH administration was calculated for TSH, TT3 and TT4. Data were analysed at $P < 0.05$. Highlights included post-TRH TT4 and TT3 concentrations having a group and week interaction ($P < 0.001$) with Control mares having higher values ($P < 0.025$) in week 12 than week 0, and Control mares in week 12 having higher values than mares in Low and High GSL groups. The TT4 FC values had a group ($P <0.001$) and group by week interaction ($P <0.001$) with week 12 Control concentrations higher ($P < 0.006$) than all other groups. Iodine concentrations decreased ($P < 0.002$) over time in GSL mares. In conclusion, feeding mares a low iodine diet with 20 and 35 mmol sinigrin/day resulted in lower serum iodine concentrations and lower TT4 concentrations responsiveness to TRH at week 12.

3.2 Introduction

The birth of congenitally hypothyroid foals has been reported in Europe [118] and North America [6]. Hypothyroid foals are called “mustard foals” in the USA, because of the association with grazing mares in pastures with wild mustard plants that contain compounds called glucosinolates (GSL) [147]. Glucosinolate containing weeds in the Western Canadian prairie region include: Argentine canola (*Brassica napus*); ball mustard (*Neslia paniculate*); dog mustard (*Erucastrum gallicum*); flixweed (*Descurainia sophia*); Polish canola (*Brassica rapa*); tumble mustard (*Sisymbrium altissimum*); wild mustard (*Brassica kaber*), and shepherd’s purse (*Capsella bursa-pastoris*); along with cultivated oilseed plants such as: canola (*Brassica napus*), rapeseed (*Brassica napus*) and mustard (*Brassica juncea*). These plants are widespread, and heat

maps have been generated showing the percentage of fields where these weeds are found in the Canadian prairies [75]. Most of these plants are present throughout the United States and many originated in Europe [75,148].

The Great Lakes area and prairie regions of North America are known for iodine deficient soil [70,109]. Plants such as kale, broccoli, brussel sprouts and mustard plants all contain GSL compounds. Low levels of GSL may have beneficial effects on health, but higher concentrations may have adverse effects [66]. Sinigrin is a bitter tasting GSL compound found in many plants including mustard. When consumed sinigrin is hydrolysed by a plant and intestinal enzyme called myrosinase into nitriles and allyl isothiocyanates. Consumption of GSL through wild and cultivated *Brassica spp.* plants may interfere with iodide uptake, because GSL like sinigrin, are metabolized into thyroid suppressing compounds such as: thiocyanates, thiouracils, isothiouracils and nitriles [112,149]. Diets containing significant amounts of GSL cause hypothyroidism in many species including: chickens, pigs, cattle, goats, sheep, buffalo, rodents and rabbits, and may affect thyroid function of their offspring because GSL metabolites cross the placenta [66,150]. Feeding high amounts of iodide along with moderate amounts of GSL ameliorates the effects of GSL on thyroid function [66,150]. Dietary factors, such as the level and types of GSL in the diet, other trace minerals, and nitrates affect the uptake of iodide by the thyroid through effects on the sodium-iodide symporter and by affecting the organification of iodide [35,66]. Different plant species may contain a variety of GSL compounds [66] and GSL containing feeds, such as canola meal and rapeseed meal, have been fed to growing horses without negative effects [141,142,151]. However, there have been no nutrition trials directly assessing the effects of GSL on serum iodine concentrations or thyroid hormones in horses.

Healthy thyroid function is essential for horses because of the central role of thyroid hormones in: thermogenesis, development, metabolism, growth, and reproduction [1]. Thyroid health in horses is related to many factors, including iodine intake [19,150]. The relationship between dietary iodine deficiency and thyroid disease in horses was recognized in the veterinary literature in 1931 and was reported to cause stillbirth, contracted tendons in foals, and goitre [70,109]. Currently, extensively managed horses are commonly supplemented with iodine through the use of iodized salt blocks and pelletized feeds; however, whether this and other management practices supply sufficient amounts of iodine to horses remains unknown [68].

Additionally, there are many gaps in our understanding of the effect of physiologic status and season on thyroid function. Fazio et al. (2012) reported intrinsic seasonal fluctuations in TT4 and TT3 levels in barren donkeys and in pregnant and non-pregnant mares [30,152]. There is little information on the response to TRH in mares in different seasons [25]. Our objective was to determine the effects of feeding a low iodine diet combined with a natural GSL (sinigrin) obtained from oriental mustard on serum iodine concentrations and thyroid hormone levels in mares. These conditions including low dietary iodine and GSL exposure were created to mimic risk factors for mares associated with the birth of foals with congenital hypothyroidism. The roles of GSL and current iodine supplementation in thyroid disease in horses needs further elucidation. Closing this knowledge gap is important to the equine industry because of the key role of the thyroid in equine health and well-being [1,19]. The hypothesis was that feeding a low iodine diet combined with the GSL (sinigrin) for 12 weeks would alter thyroid hormone levels and decrease serum iodine levels in mares.

3.3 Material and Methods

3.3.1 Mare Management

University of Saskatchewan's Institutional Animal Care and Use Committee approved the animal use in this study. Healthy non-pregnant light breed mares (n=19) ranging in age from 2 to 14 years [8.3 ± 0.8 years (mean± SD)] were housed in dry lots with straw bedded shelters. The feeding trial was performed for 12 weeks beginning in late September to mid-December 2017. A physical examination was performed on each mare (temperature, pulse, respiration, mucous membrane color, auscultation, palpation of lymph nodes and thyroid, evaluation of manure consistency) at the beginning of the trial. Mares were weighed at the start and end of the trial.

3.3.2 Feed palatability and source of GSL

A feed palatability trial was performed prior to the start of the experiment. A 500 g mustard meal composed of 450 g of oats combined with 50 g (10%) oriental mustard powder (OMP) and molasses was offered to each mare daily for week and a half. Six out of nineteen

mares refused to eat feed containing the OMP. Eight mares consumed all of the oriental mustard oats mixture, whereas five mares ate half. Therefore, mares that refused the meal were assigned to Control (n=6) group, whereas mares that would consume the OMP mixture were randomized into the High GSL (n=6) and Low GSL (n=7) group.

Oriental mustard powder (Sakai Spice, 4201 2nd Ave North, Lethbridge, Alberta, Canada T1H0C8) was utilized as the source of GSL. Glucosinolate content of the OMP, oats and hay used in the study was analyzed at the Lipids Quality and Utilization Lab, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK Canada, and only the OMP was found to contain the GSL, sinigrin, at 128.9 mmol / kg. Sinigrin is a GSL whose secondary metabolites include isothiocyanates and nitriles.

3.3.3 Diets

An isocaloric and isonitrogenous diet was formulated for each group based on grass hay, oats, and oriental mustard powder in the GSL groups. A small amount of liquid molasses was used to mix the oriental mustard powder into the concentrate. Table 3.1 shows the feed composition of the diets and the daily intake of nutrients. The mares had continuous access to trace mineral salt blocks (10:10 Agri-Blok, Canadian Agri-Blend Inc., 8702A 98th Street Morinville Industrial Park, Morinville, Alberta, Canada T8R 1K6) which contained 4800 mg/kg iron, 2500 mg/kg copper, 10,000 mg/kg zinc, 8000 mg/kg manganese, 30 mg/kg cobalt, 200 mg/kg iodine, and 30 mg/kg selenium until the beginning of the feeding trial. The mares were trained to go to an individual bucket where they were tied up and fed their concentrate ration. Any residual feed left in the bucket was weighed back. The hay was weighed for the group, divided and fed in individual small diamond hay nets.

At trial initiation, the oat ration was fed twice daily and increased gradually over a one-week period until a total of 1.8 kg DM oats per day was reached. Mares in the Control group (n=6) were fed trace minerals and iodized salt to meet their minimal requirements for minerals as per NRC (2007). For the first 6 weeks, a similar percentage of OMP from the palatability trial was included in the concentrate of the GSL groups as follows: mares in the Low GSL group (n=7) received 110 g (5.8%) per day of OMP (12.5 mmol GSL), and mares in the High GSL group (n=6) received 220 g (11%) per day of OMP (25 mmol GSL) in the concentrate. The OMP

level was increased to 160 g or 8.2% OMP (20 mmol GSL) in the Low GSL group and to 280 g or 13.5 % OMP (35 mmol GSL) in the High GSL group from 6 weeks until the end of the feeding trial. It is worth mentioning that a third attempt was performed to increase the mustard level to 25 and 50 mmol GSL in the Low and High GSL group, respectively; however, this resulted in the rejection of some or all of the concentrate mixture in 11/13 mares.

3.3.4 Thyroid releasing hormone (TRH) response tests

The TRH stimulation test was initiated between 8:30 am and 10:00 am on all mares at week 0 and 12 weeks. A baseline jugular sample was drawn followed by the intravenous administration of 1 mg of TRH (TRH, Sigma Aldrich Chemical Company, 2149 Winston Park Drive, Oakville, Ontario, Canada L6H 6JH). Blood samples were then obtained using sterile tubes without anticoagulant at 45 minutes, 2 hrs and 4 hrs post TRH injection. Blood was refrigerated overnight, centrifuged, serum separated and stored at -20 C.

3.3.5 Definitions

The definition of the euthyroid state at the beginning of the trial was basal thyroid hormone levels within the basal adult reference range used by the Animal Health Diagnostic Center Endocrine Laboratory at Cornell University: TT4 (12.87 – 38.6 nmol/L), TT3 (0.408 – 1.088 nmol/L), and for TSH the range reported by Breuhaus (2002) (0.03 – 0.97 ng/mL), or a response to TRH administration within the range reported by Breuhaus (2011) which for TT4 was 1.3 – 3.8 fold increase over the Pre-TRH TT4 level at 4 hours Post-TRH administration or for TT3 was a 1.1 – 10.3 fold increase over the Pre-TRH TT3 level at 2 hours Post-TRH administration [19,111]. The definition of an ‘adequate response’ to TRH was a doubling of the TT4, TT3 and TSH concentrations compared to basal levels according to Breuhaus (2011) [19]. Fold change (FC) is the ratio of Post-TRH/basal hormone concentration at the same Post-TRH time points for each hormone.

3.3.6 Thyroid hormones

Thyroid hormones, total thyroxine (TT4) and total triiodothyronine (TT3) concentrations were assayed using the Immulite method (Siemens Immulite Canine TT4, and TT3, 1577 North Service Road East, Oakville, Ontario, Canada L6H 0H6) validated for use with equine serum at the Endocrine Laboratory, Prairie Diagnostic Services, Saskatoon, SK. The analytical sensitivity of the TT4 assay was 0.15 nmol/L. Specificity data from the manufacturer identified 100% cross-reaction with L-thyroxine, 55% with D-thyroxine, 16% with tetraiodothyroacetic acid, and 3.2% with triiodo-L-thyronine. The intra-assay coefficients of variation for TT4 were 10.6%, 6.4% and 5.2% for reference sera with a mean TT4 concentration of 10.55, 22.4 and 42.0 mmol/L, respectively. The analytical sensitivity of the TT3 Immulite assay is 0.54 nmol/L. Specificity data from the manufacturer identified 100% cross-reaction with triiodo-L-thyronine, 100% with triodo-D-thyronine, 1.3% with tetraiodothyroacetic acid, and 0.7% with triiodothyroacetic acid. The inter-assay coefficients of TT3 were 2.1%, 3.5% and 0.9% for reference sera with mean triiodothyronine concentrations of 1.1, 3.1 and 6.0 nmol/L, respectively. Laboratory personnel were blinded to the treatment group identity of the samples. Reference ranges for thyroid hormones in adult horses were obtained from the Animal Health Diagnostic Center Laboratory at Cornell University, Ithaca, NY, 148523, previously described in the Definition section. The concentrations of TT4 and TT3 were determined basal, at 2 hr after TRH injection for TT3 (2hr post-TRH) and at 4 hr after TRH injection for TT4 (4hr post-TRH).

3.3.7 TSH

The TSH assay was conducted by Donald L. Thompson at Louisiana State University, Baton Rouge, LA using a RIA assay validated for horses [153]. Briefly, TSH was measured by a double-antibody RIA based on anti-TSH antiserum (AFP-33812) and highly purified equine TSH (AFP-5144B) supplied by A. F. Parlow (National Hormone and Pituitary Program, Harbor-UCLA Medical Centre, Torrance, CA) [153]. The intra-assay coefficient of variation was 6.0% and the inter-assay coefficient of variation was 7.2%. The lower and upper reference range for TSH used was 0.03 – 0.97 ng/mL, which is from Breuhaus (2002) for horses from three to nineteen years of age [111]. Thyroid stimulating hormone was measured in at baseline and 45-minute Post-TRH sampling time.

3.3.8 Serum Iodine

Serum iodine concentration was measured at the Animal Health Diagnostic Laboratory at University of Guelph, Guelph, ON, using an ICP-MS method in serum samples obtained at week 0 and week 12 of the study. The detection limit of ICP-MS method for serum iodine was 4 µg/L. The reference ranges for serum iodine were <10 µg/L deficient, 11- 19 µg/L marginal, 20 - 49 µg/L adequate, >50 µg/L high. Reference ranges were based on the published values from Puls (1994) [41] and Mochizuki et al. 2016 [8]. An 'adequate' serum iodine level was defined as a serum iodine level at or above the low end of the healthy adult reference range (20 µg/L) [41].

3.3.9 General Data Analysis

For all tests, data were considered statistically significant if $P < 0.05$. The TT4, TT3, TSH, and iodine data were analysed for normality (Shapiro-Wilk test), Kruskal-Wallis, Wilcoxon pairs test, and Fisher's Exact Tests using proprietary software (StatPlus 2, Analyst Soft, 340 South Lemon Ave. #3010, Walnut, California, USA 91789). Descriptive data is reported by Group for TT4 [mean ± SD (range)], and TT3, TSH and iodine [median (quartiles)] at week 0 and week 12. Changes in mare weight from week 0 to week 12 were evaluated using a Wilcoxon Pairs Test. Comparisons were made within groups using Kruskal-Wallis to examine the change in serum iodine from week 0 to week 12. A generalized estimating equation analysis (GEE) was performed for the three hormones (TT4, TT3 and TSH) and iodine concentrations using proprietary software (SPSS version 24a, IBM, 1 New Orchard Road, Armonk, New York, USA 10504-1722). The main effects in the GEE model were 'Week' (week 0 and week 12) and 'Group' (three treatments; Control, Low GSL and High GSL) and interaction between "Week" and "Group" variables were included in the model. Comparisons between Groups were made at each time point and changes over time within the same group were evaluated. Parameters examined for each hormone (TT4, TT3, and TSH) using the GEE included: basal level, post-TRH administration level and FC. The post hoc analysis was the Least Significant Difference (LSD) which included pairwise comparisons of the groups between and within each time points. Serum iodine concentrations at week 0 and week 12 were compared with Group and Week as main effects and their interaction in the model. The post-hoc test for serum iodine level was LSD.

Comparisons were made between the proportion of mares in each group at week 0 and week 12 in basal TT4, TT3, TSH and serum iodine concentration in the normal reference range (at or above the lowest value of the respective adult reference range); and comparisons were made between the proportions of mares with an 'adequate response' i.e. two-fold or greater increase from the baseline TT4, TT3 and TSH level to the 4hr TT4, 2hr TT3, and 45 min TSH level respectively, using Fisher's Exact Tests.

3.4 Results

3.4.1 General

All mares were considered to be healthy at the initiation of the trial based on physical examination findings. The mares were determined to be euthyroid based on basal TT4 and TT3 concentrations or an increase after the TRH injection at 4hr TT4 and 2hr TT3, which were within the reference range for each hormone [19]. We found at week 0 that 3/19 mares did not have a minimum FC of 1.1 in the 2hr TT3 level and 1/19 mares did not have a minimum FC of 1.3 for the 4hr TT4 level; however these mares had high basal levels that were within the normal reference range for TT3 and TT4. There were 18/19 mares with serum iodine level in the reference range at week 0, and one mare in the Control group had a serum iodine concentration of 19 μg/L, which was close to the low reference range value for healthy horses of 20 μg/L [111]. There were no significant changes in the mares' weights from week 0 to week 12. According to the corresponding type of feed of each group, feed consumption was the same between control and GSL groups during the feeding trial.

3.4.2 TT4

There were no differences (mean nmol/L ± SD) in basal TT4 concentrations at week 0: Control (22.6 ± 4.0); Low GSL (27.2 ± 6.8); High GSL (23.6 ± 5.4); but there was a significant effect of Week ($P = 0.022$) with lower basal TT4 values by week 12 found in Control (20.8 ± 9.4); Low GSL (23.2 ± 8.0); and High GSL (20.8 ± 4.2) mares. Regarding post-TRH TT4 concentrations, there was an effect of Group and Week ($P < 0.001$). The post-TRH TT4 concentrations (mean nmol/L ± SD) at week 12 for Control (45.6 ± 13.1) were higher compared

to: week 0 Control (35.1 ± 9.2) (P <0.001), week 12 Low GSL (31.7 ± 10.9) (P = 0.025) and week 12 High GSL (25.8 ± 2.5) (P <0.001); but there were no differences at week 0 between Control, Low GSL (35.7 ± 8.5) and High GSL (35.4 ± 8.5) groups.

There was a significant Group (P <0.001) and Group and Week interaction (P <0.001) in TT4 FC [median nmol/L ± median standard error (SE) (quartiles)], with week 12 Control group [2.2 ± 0.1 (1.8, 2.7)] values higher than all other groups as follows: Control week 0 [1.6 ± 0.05 (1.4, 1.7)] (P <0.001), week 12 Low GSL [1.3 ± 0.03 (1.2, 1.4)] (P <0.001) and week 12 High GSL [1.2 ± 0.04 (1.1, 1.4)] (P <0.001); week 0 Low GSL [1.4 ± 0.06 (1.1, 1.4)] (P < 0.001) and week 0 High GSL [1.5 ± 0.1 (1.3, 1.7)] (P=0.006). The scatter plots for the basal TT4, Post-TRH TT4 and TT4FC data at week 0 and week 12 are shown in Figure 3.1 to 3.3.

3.4.3 TT3

There were no differences [median nmol/L ± SE (quartiles)] in basal TT3 concentrations at week 0: Control [1.0 ± 0.1 (0.6, 1.4)]; Low GSL [1.2 ± 0.1 (0.8, 1.6)]; High GSL [0.5 ± 0.2 (0.4, 0.9)]; and at week 12: Control [1.5 ± 0.3 (1.2, 1.7)], Low GSL [1.1 ± 0.1 (0.9, 1.3)] and High GSL [1.3 ± 0.1 (1.0, 1.4)] groups. Regarding the post-TRH TT3 concentrations there was an effect of Group (P = 0.001) and a Group by Week interaction (P = 0.001). There were higher post-TRH TT3 concentrations in week 12 Control [median nmol/L ± SE (quartiles)] [6.61 ± 0.4 (5.6, 7.6)] versus: week 0 Control [2.1 ± 0.2 (1.6, 3.1)] (P <0.001), week 0 Low GSL [1.8 ± 0.2 (1.3, 3.2)] (P <0.001); week 12 Low GSL [1.82 ± 0.2 (1.5, 2.6)] (P <0.001) and week 12 High GSL [1.83 ± 0.1 (1.6, 2.1)] (P <0.001); with no effect on the week 0 High GSL group [2.5 ± 0.7 (2.4, 6.3)]. Regarding TT3 FC there was an effect of Group (P = 0.018) and Group and Week interaction (P = 0.023). The week 12 Control group [median ± SE (quartiles)] FC [4.1± 0.6 (2, 6.2)] was greater than the week 12 Low GSL FC [1.6 ± 0.1 (1.5, 1.8)] (P = 0.02); week 12 High GSL FC [1.5 ± 0.1 (1.1, 1.7)] (P <0.01); and week 0 Low GSL FC [2.1 ± 0.1 (1.1, 1.5)] (P = 0.028) with no difference for week 0 Control FC [2.6 ± 0.4 (1.7, 3.5)], and week 0 High GSL FC [5.6 ± 1.5 (3.1, 8.9)].

3.4.4 TSH

There were no differences between weeks or groups in TSH for basal, 45-minute post-TRH or FC. Table 3.2 shows the [median ng/mL ± SE (quartiles)] TSH concentrations in basal, post-TRH and FC values at week 0 and week 12.

3.4.5 Serum Iodine

Serum iodine concentrations [median µg/L ± SE (quartiles)] at week 0 versus week 12 were: Control [23.5 ± 0.8 (22.25, 0.85) versus 37.5 ± 1.2 (29.5, 44.0)]; Low GSL [26 ± 0.7 (23.5, 27.5) versus 15 ± 0.8 (14.5, 20)] and High GSL[22 ± 0.6 (21.25, 25) versus 16.5 ± 0.4 (14.5, 17.8)]. There was a significant effect of Group ($P < 0.001$) and a Group by Week interaction ($P < 0.001$) on iodine concentrations. Control mare serum iodine concentrations had a positive change from week 0 to week 12, and by week 12 control mare concentrations were higher than all other groups ($P < 0.002$) at all time points. Low and High GSL Group's serum iodine concentrations had a negative change from week 0 to week 12 with the change (week 0-week 12) [median µg/L ± SE (quartiles)] in serum iodine concentrations as follows: Control [-11.5 ± 1.9 (-21.0, -5.0)]; Low GSL [9.0 ± 9 (8.0, 10.0)]; and High GSL [7.5 ± 0.4 (8.0, 5.5)].

3.4.6 Fisher's Exact test:

A comparison of the proportion of mares with adequate basal TT4, TT3, and TSH was statistically similar at week 0 between groups. A comparison of the proportion of mares in a group that achieved an adequate TT4 response (doubling of basal TT4 concentration by post-TRH) at 12 weeks was different ($P = 0.004$), and included 4/6 Control, 0/7 Low GSL, and 0/6 High GSL mares. A comparison of the proportion of mares in a group with a doubling of their basal TSH value at post-TRH at week 0 versus 12 was: Control 5/6 versus 1/6; Low GSL 4/7 versus 6/7; and High GSL 6/6 versus 3/6, respectively, with $P = 0.055$. Regarding serum iodine concentrations, a comparison of the proportion of mares in a group at week 0 having serum iodine concentrations in the reference range were not different with 5/6 control mares, 7/7 Low GSL mares and 6/6 High GSL mares having adequate levels. At week 12 there was a difference ($P = 0.0005$) in the proportion of mares with iodine concentrations in the reference range with 6/6 Control mares, 2/7 Low GSL mares, and 0/6 High GSL mares having adequate serum iodine concentrations.

3.5 Discussion

The goal of the study was to create conditions such as a low dietary iodine and GSL exposure, which are reported to be risk factors for mares that deliver foals with congenital hypothyroidism and determine the effects on serum iodine and thyroid hormones concentrations [6,118,147]. We partially accept our hypothesis, as our study showed that feeding a low iodine diet in combination with the GSL sinigrin at 20 and 35 mmol/day for 12 weeks to non-pregnant mares had no effect on body weight, but lowered serum iodine concentrations and affected the thyroid secretion of TT4 in response to TRH administration with no effect on TT3 compared to Control group; however, FC TT4 was not different between High and Low GSL groups. It is worth mentioning that due to the study design, we are unable to draw final conclusions as to the relative contribution of low iodine supplementation versus the addition of GSL to the diet. This study would have been strengthened by the addition of a Control group without sinigrin, and a GSL group with iodine supplementation; however due to financial constraints this was not performed. Nevertheless, the combination of low dietary iodine and GSL may have negative effects on pregnant mares and neonates because of the essential role of thyroid hormones and iodine during embryonic, fetal and early life development based on studies performed in other domestic animals, such as goats, but requires further study.

The amount of GSL fed in this study (35 mmol/day) could be easily consumed by a horse as 12.2 mg GSL /g dry weight of whole wild mustard seed has been reported and over 130 μmol/g of cultivated oil seed [154,155]. Canola meal (*Brassica napus*), a variety of plant selected for low GSL content, has been widely used in rations for dairy and beef cattle, swine, poultry and fish production due to the high protein quality [66]. Cymbaluk (1990), Oliviera et al, (2001) reported feeding different levels of canola meal in growing foals with no adverse effects, such as decrease feed intake and delayed in growth rate, in a 60 - 75-day period [141,142]. This suggests that similar to our study, relatively short periods of GSL feeding to horses do not affect health in terms of weight. The cost benefit of adding various GSL containing ingredients to equine feeds has not been determined, as the tolerance to different GSL have not been fully described. It is worth mentioning that content and composition of GSL varies by plant species and is affected by climatic conditions, thus, the GSL type and content from the same plant species may be different every year. Experimentally this means GSL content in feed ingredients must be analyzed. In the present study, we had concerns over the palatability of the GSL because horses are known to be

selective eaters, and decreased intake or feed refusal may occur with high amounts of GSL in the diet [66]. Our initial objective was to increase the feeding of the GSL sinigrin contained in the OMP (*Brassica juncea*) to 50 mmol GSL per day for the Low GSL group and 100 mmol GSL per day for the High GSL group, which was based on previous reports in cattle, where it was reported that a concentration of 100 mmol GSL was able to induce thyroid dysfunction [66]. Nonetheless, an increase in the amount of OMP more than 35 mmol sinigrin per day lead to a decrease in the voluntary intake and rejection of the concentrate mixture in the majority of the GSL mares; however, one mare in each of the Low GSL and High GSL groups tolerated the higher concentration, suggesting that under natural conditions individual variability in voluntary intake and tolerance for GSL may make some horses more likely to consume mustard plants than others. Furthermore, a concentration of 20 – 35 mmol/day of sinigrin was adequately tolerated by the Low and High GSL groups, respectively, until the end of the feeding trial and there was no change in feed consumption.

There is little information regarding the tolerance to and effects of sinigrin, which is metabolized to isothiocyanates, and nitriles, on equine thyroid hormone function. Isothiocyanates have been reported to reduce the thyroid's ability to take up iodine, synthesize thyroid hormone, and release thyroid hormones [112,132]. We report that sinigrin at the level fed in this study combined with low dietary iodine conditions influences the thyroid response to TRH. In this study there was a failure in both GSL groups to respond adequately to TRH stimulation as shown by a lower TT4 and TT3 FC, compared to Control group mares at week 12 in spite of having similar Post-TRH TSH levels. Figure 1 shows there is very little dispersion of the T4 data in the GSL groups. The higher response by Control mares at week 12 may be attributed to seasonal factors, but this would be in contrast to the findings of other research groups that concluded there was no effect of season on TRH response in horses [25,156]. Due to the small group size in this study and our experimental design we cannot be certain if the increase in Control Group mare's response to the TRH stimulation test was due to chance, a seasonal effect, a result of the iodine supplemented diet in the 12-week period, an interaction between factors or due to a combination of factors. Other authors have reported seasonal changes in basal secretion of TT4 [30,33,152].

Fazio et al. (2016) reported intrinsic seasonal changes in thyroid hormone synthesis in pregnant and non-pregnant mares in basal TT3 and TT4 [30]. Johnson (1986) reported highest concentrations of TT4 in horses during October and November, whereas TT3 concentrations were

high through December to May and lower from July to October [33]. There are a variety of extrinsic factors that influence thyroid hormones synthesis such as: age, sex, feed deprivation, diets containing high energy and protein content, stress, trace minerals, and treatment with medications such as phenylbutazone and corticosteroids [1,19,25,35,112]. One study in a region known for endemic goiter in human and domestic animals showed that adult horses without supplementation of iodine and no exposure to *Brassica* plants had basal TT4 levels higher than horses in the non-endemic area and were without clinical signs of hypothyroidism [27]. The authors concluded that the horses in this region were resilient and had developed compensatory mechanisms for low environmental iodine and were not hypothyroid [27]. Another author reported that foals with goiters and mares on the same farm had low TT4 but TT3 levels were within the normal range in an area with iodine deficient soil [108]. Medica et al. (2011) reported that under similar environmental, management, and nutritional conditions that pregnancy status in mares played a dynamic role on thyroid hormone patterns and may be very important in early embryonic growth and development [27]. Indicating that substances such as GSL that may alter maternal thyroid function should be avoided throughout pregnancy in mares.

Regarding TSH, the basal and Post TRH TSH concentrations were not significantly different compared to Control mares, indicating that pituitary function was adequate and the lack of change in the Pre-TRH TSH level indicates that primary hypothyroidism was not induced after a three-month exposure to sinigrin. In cases of primary hypothyroidism, basal TSH and post TRH responses are dramatically increased [19]. However, in horses, primary hypothyroidism has only been achieved experimentally by the administration of highly purified propylthiouracil (PTU), a well-known synthetic goitrogen used in human medicine to treat hyperthyroidism. The administration of PTU has been used to induce experimental hypothyroidism in rats, cattle, sheep and horses [66,150]. We concluded that sinigrin when fed for 12 weeks in this study along with a low iodine diet affected thyroid function, because of the effect on the Post TRH response at week 12 in the GSL groups. A diet formulated with a higher amount of sinigrin, or a more potent goitrogenic GSL compound fed for a longer period of time, along with a diet deficient or low in iodine would likely be required, to induce primary hypothyroidism. Other GSL whose metabolites are much more potently goitrogenic, may induce changes in TSH concentrations in a shorter period of time in mares as they do in other species [66].

It has been reported in other farm animals that chronic exposure to secondary metabolites of GSL such as isothiocyanates, thiocyanates and oxazolidithione decreases iodine uptake by the thyroid thus causing thyroid dysfunction [66]. There are species differences in their sensitivity to the effects of GSL. In cows and pigs, a concentration between 10 to 11 mmol/kg GSL of the diet can induce iodine deficiency, whereas in the sheep a concentration ≥ 4.22 mmol/kg GSL of the diet causes iodine deficiency in pregnant ewes and affects thyroid weight and thyroid histology in newborn lambs [150]. The prevalence of goitre and thyroid insufficiency in newborn lambs was reported in pregnant ewes fed different *Brassica* family plants (rye grass, radish, and rape) with or without iodine supplementation [66,150]. The ewes fed radish forage (total GSL per diet: 3.0 mmol/kg, mainly glucobrassicin) and rape (total GSL per diet: 7.45mmol/kg, mainly progoitrin) failed to respond adequately to a TRH stimulation test [150]. The lambs born to ewes fed rape without iodine supplementation resulted in a higher incidence of goitre compared to ewes fed radish forage, and both groups had decreased TT4 following administration of TRH [150]. The results of this particular study in sheep were used to justify why no additional iodine was added to the mares in the GSL diet groups, as the threshold for protection from the effects of GSL by iodine supplementation in horses is not known [66]. The findings of this study showed that the type, and concentration of GSL, along with iodine supplementation all play a key role in the development of fetal goitres during pregnancy in sheep, and illustrated that in cases with no evident goitres, thyroid hormone synthesis is still affected [150].

Serum iodine concentrations decreased a median of 9 µg/L in the Low GSL group, and 7.5 µg/L in the High GSL group by week 12. The result was a decrease in serum iodine from the normal to the marginal range according to Puls (1994) in 5/7 mares in the Low GSL group and 6/6 mares in the High GSL group [41]. The week 0 serum iodine levels of 18/19 mares were within the reference range reported by Puls (1994) [41]. This was interpreted to mean that there was no pre-existing iodine deficiency in the mares based on these values. As the groups were not different in iodine levels at week 0, we concluded that the decrease in iodine was a reflection of the combined effect of the GSL, and the non-iodine supplemented diet, which then resulted in an impaired response to TRH. The GSL group diets were low in iodine content but were not iodine free. The NRC requirement for iodine in mature horses whose mean weight is 464 kg is 3.2 mg day. Based on the values reported in Equine Clinical Nutrition (2013) for iodine content in hay and grain we calculated that the intake of iodine on a dry matter basis in the base ration would be

in the range of 1.6 – 3.3 mg of iodine (50% to 103% of the recommended value) [35]. A non-iodine supplemented diet for 12 weeks was deemed unlikely to produce this effect on the thyroid response to the TRH alone; however the experimental design does not allow us to draw firm conclusions in this regard [150].

There are no studies addressing the effect of iodine supplementation on thyroid responsiveness in horses; however in euthyroid human patients, high iodine supplementation decreases TT4 and TT3 and mildly increases TSH [157]. The Control mare's serum iodine values were maintained within the reference range for healthy horses at both time points [41]. Studies in humans have shown that iodine supplementation to euthyroid people twice the recommended daily intake for a 2-month period did not modify thyroid function by increasing or decreasing basal levels of free TT4 and TT3 [157]. Excess iodine supplementation can result in a reduction in thyroid hormone synthesis through a regulatory mechanism in which there is a temporally protection against an excessive production of TT3 and TT4 when the thyroid gland is exposed to a sudden large amount of iodine [157].

The sensitivity to the effects of GSL by adult horses compared to equine fetuses is unknown. The demonstration that GSL suppressed serum iodine in non-pregnant mares likely means that dietary GSL would affect pregnant mares and may also impair embryonic development and fetal thyroid function. The GSL metabolites have been reported to cross the placenta and affect fetal thyroid function in many species; however, future research should be performed to confirm this relationship in pregnant mares [66,147].

Table 3.1: Ingredient and nutrient composition of diet groups based on dry matter: Control, Low and High glucosinolate (GSL) (upper) and nutrient composition and daily intake of the mares (lower).

Ingredient and Nutrient Composition of Diets Fed to Non-Pregnant Mares

Ingredient	Intake (daily)	Control Wk 0 - 12	Low GSL Wk 0 - 6	Low GSL Wk 6 - 12	High GSL Wk 0 - 6	High GSL Wk 6 - 12
Mixed Grass Hay	kg	7.14	7.14	7.14	7.14	7.14
Oats	kg	1.78	1.78	1.78	1.78	1.78
Mustard Meal Powder	gm	-	110	160	220	280
Molasses, beet sugar	ml	20	160	160	200	200
Weathergard 22	gm	50	-	-	-	-
Iodized salt	gm	25	-	-	-	-

Nutrient Composition (daily intake)

Nutrient	Amount	NRC*	Control Wk 0 - 12	Low GSL Wk 0 - 6	Low GSL Wk 6 -12	High GSL Wk 0 - 6	High GSL Wk 6 - 12
Digestible energy	Mcal	16.65	18.79	19.29	19.29	19.42	19.42
Crude protein	gm	630	994	1046	1065	1091	1113
Calcium	gm	20	58	50	50	50	51
Phosphorus	gm	14	21	17	17	18	19
Selenium	mg	1.0	1.3	0.8	0.8	0.8	0.8
Iodine	mg	3.5	6.6	0.3	0.3	0.3	0.3

*NRC requirements for 500 kg non-pregnant mares 2007

Table 3.2: Serum TSH concentrations (ng/ml) [median ± SE (quartiles)] levels in basal, 45-minute post-TRH samples and the Fold Change (Post-TRH divided by Pre-TRH level) in: Control, Low and High glucosinolate (GSL) group mares at week 0 and week 12.

TSH		**Week 0**	
Group	**Pre-TRH**	**Post-TRH**	**FC**
Control	0.7 ± 0.12 (0.2 - .97)	1.66 ± .49 (0.6 – 3.4)	3.1 ± 0.2 (3.1 – 3.2)
Low GSL	0.6 ± 0.1 (0.2 – 1.24)	1.96 ± 0.4 (0.6 – 3.1)	2.5 ± 0.8 (1.6 – 3.04)
High GSL	0.48 ± 0.1 (0.06 – 0.7)	1.5 ± 0.23 (0.5 – 2.2)	4.0 ± 0.5 (2.7 – 5.2)
		Week 12	
Group	**Pre-TRH**	**Post-TRH**	**FC**
Control	0.57 ± 0.14 (0.50 – 1.38)	1.01 ± 0.29 (0.71 – 2.34)	1.2 ± 0.1 (1.7 – 1.9)
Low GSL	0.5 ± 0.05 (0.4 – 0.8)	1.15 ± 0.3 (0.9 – 3.2)	2.5 ± 0.1 (2.1 – 2.7)
High GSL	0.4 ±0.06 (0.3 – 0.6)	1.2 ± 0.31 (0.2 – 2.4)	2.2 ± 0.2 (1.9 – 3.0)

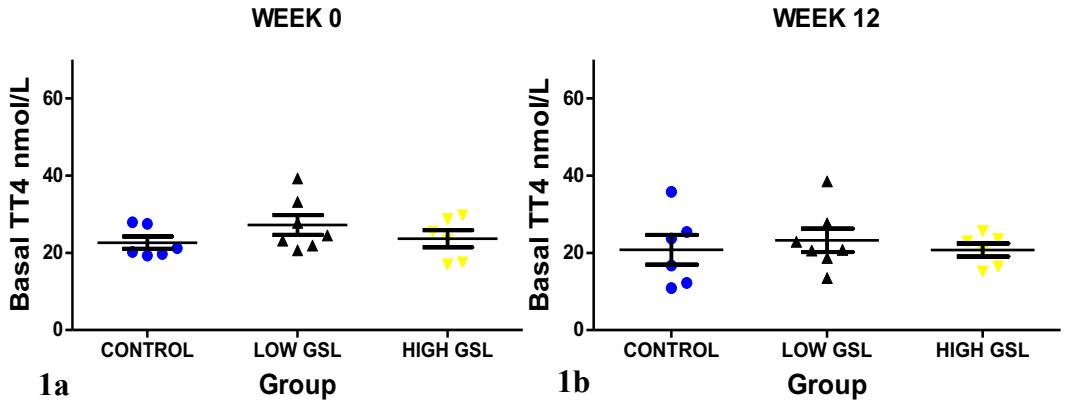

Figure 3.1. Scatter plot of serum total thyroxine (TT4) (nmol/L) concentrations in Control, Low and High glucosinolate (GSL) groups in: 1a) Basal TT4 concentrations at week 0; 1b) Basal TT4 concentrations at week 12. The long horizontal line represents the mean and short horizontal lines the SEM.

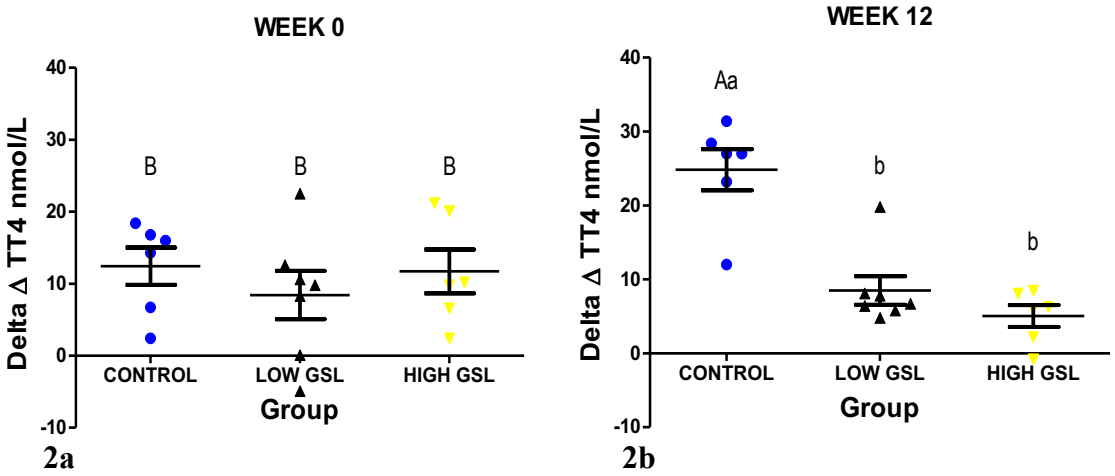

Figure 3.2. Scatter plot of serum total thyroxine (TT4) (nmol/L) concentrations in Control, Low and High glucosinolate (GSL) groups showing: 2a) the Delta in TT4 concentrations at week 0; and 2b) at week 12. The long horizontal line represents the mean and short horizontal lines the SEM. Upper case letters represent differences between groups over time and lower case letters represent differences between groups in the same time point.

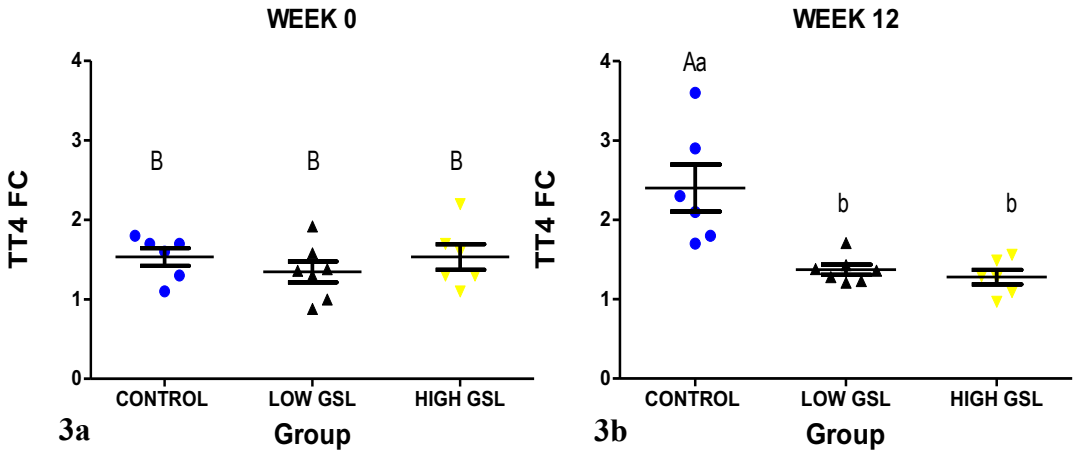

Figure 3.3. Scatter plot of serum total thyroxine (TT4) (nmol/L) concentrations in Control, Low and High glucosinolate (GSL) groups in: 3a) TT4 Fold Change at week 0; 3b) TT4 Fold Change at week 12. The long horizontal line represents the mean and short horizontal lines the SEM. Upper case letters represent differences between groups over time and lower case letters represent differences between groups in the same time point.

CHAPTER 4

A Field Study of Serum, Colostrum, Milk Iodine and Thyroid Hormone Concentrations in Post-partum Draft Mares and Foals

TRANSITIONAL PAGE

Authors: M.F. Lopez-Rodriguez[a], N.F. Cymbaluk[a], T. Epp[a], B. Laarveld[b], M. Thrasher[a] and C.E. Card[a]

Institutional affiliations: [a]Dept. Large Animal Clinical Sciences, [b]Dept. of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

Source of funding: Thanks to Hilltopper Clydesdales of Saskatoon, SK for their participation in this research. The research was funded by the Mark and Pat DuMont Equine Orthopedics Research Fund of the Western College of Veterinary Medicine.

This chapter concerns the characterization of colostrum, milk and serum iodine levels in healthy post-partum mares and foals. The effect of an inadequate iodine supplementation in the diet during pregnancy is still unknown due to the lack of robust information regarding normal iodine reference values in foals' serum, as well as milk and colostrum samples from post-partum mares. Mares in the study received free choice iodine supplementation during pregnancy and post-partum. Iodine in serum, colostrum and milk were obtained between one to four hours post-foaling (day 0) and ten days later. The values were compared with thyroid hormone concentrations. In species with an epitheliochorial placentation, such as the horse, maternal thyroid hormone transfer to the late term fetus is believed to be minimal, if any. For that reason, maternal iodine transfer to fetal circulation is essential for normal fetal thyroid hormone synthesis.

4.1 Abstract

Iodine, thyroxine (T4) and triiodothyronine (T3) are required for normal fetal growth, maturation and neonatal survival. There is a lack of robust information on iodine levels found in colostrum, milk and serum of mares and foals following a healthy pregnancy. Our objective was to characterize colostrum, milk and serum iodine levels in healthy post-partum mares and foals (n = 10) and explore relationships with thyroid hormone concentrations. Colostrum, milk and jugular blood samples from draft breed mares and foals with an estimated average iodine daily intake of 39 mg per mare during pregnancy were obtained at day 0 (foaling date) and/or 10 days later. Parameters studied were: mare basal concentrations of serum: TT3, TT4, iodine; iodine in colostrum at day 0 and milk iodine (day 10); foal basal TT3, TT4 and serum iodine (day 0 and 10). Median ± median error colostrum iodine levels (165 ± 15.1 µg/L) were higher than milk (48 ± 5.6 µg/L) (*P* =.007) levels. Median ± median error foal serum iodine (268.5 ± 7.6 µg/L), TT4 (1225 ± 47.8 nmol/L) and TT3 (14.2 ± 1.1 nmol/L) at foaling date were higher than at 10 days (70 ± 3.6 µg/L; 69.6. ± 20.4 nmol/L; 5.4 ± 0.3 nmol/L, respectively). In conclusion, equine mammary tissue concentrates iodine beyond plasma levels, making colostrum and milk a significant source of iodine. Foal serum iodine levels are high in the neonatal period, and are positively correlated with TT4, which is important for neonatal adaptation.

4.2 Introduction

In all mammalian species, adequate levels of iodine are required to support synthesis of thyroid hormones such as thyroxine (T4) and triiodothyronine (T3), which are critical for normal intrauterine growth and fetal maturation [24]. Thyroid hormones are also involved in neonatal physiological processes, such as thermogenesis, and are essential for extrauterine life [11]. The epitheliochorial placenta of some domestic animals, such as sheep and horses, is reported to be impermeable to maternal thyroid hormones after the second trimester of pregnancy [21]. Nonetheless, iodide transfer from the maternal to fetal circulation through the placenta is necessary for active fetal thyroid hormone synthesis [11]. There is a lack of robust information on iodine levels found in mare colostrum, milk and serum of mares and foals, and little understanding of how iodine levels relate to thyroid hormone concentrations. Furthermore, while it is known that colostrum and milk provide essential nutrients, including minerals, to the

newborn foal, it has not been determined if mare colostrum is a source of iodine [158]. Puls (1994) reported that an adequate concentration of iodine in mare's milk is between 10 to 16 ug/L [41], whereas, milk iodine levels between 4 to 42 ug/L have also been reported [159]. To our knowledge there is little information regarding iodine levels in equine colostrum versus milk along with serum iodine concentrations in neonatal foals. The aim of the study was to describe levels of iodine in serum, colostrum and milk samples in relationship to thyroid hormone concentrations from mares and their foals following a healthy pregnancy. Information regarding iodine concentrations in equine serum, colostrum and milk is needed for good nutritional management and will assist in the detection of trace mineral deficiencies, which affect the development of the equine neonate.

4.3 Material and Methods

4.3.1 Mare Management

The present study was approved by the University of Saskatchewan's Institutional Animal Care and Use Committee. Healthy Clydesdale mares (n=10) ranging from 5 to 13 years with a mean age of 8.9 ± 2.7 years were bred by live cover. Mares had access to timothy grass/alfalfa hay in large round bales ad libitum for the entire pregnancy. The average weight of the mares was 680 kilograms (kg). Mares had free access to a combination of a commercial granulated equine vitamin and mineral supplement (Masterfeeds, 1020 Hargrieve Road London, Ontario, Canada N6E1P5) mixed with 150 mg/kg iodized salt (Ceres Industries, Site 603, Box 10 RR#6 Saskatoon, SK, Canada S7K3J9) at a ratio of 1:1, respectively. A 20 kg cobalt iodized salt block (iodine: 200 mg/kg; cobalt: 100 mg/kg, Ceres Industries, Site 603, Saskatoon, SK S7K3J9) was placed in the pen every month until foaling. In addition, all mares were group-fed approximately 5.1 kg dry matter (DM) oats once per week per mare (the equivalent of 0.73 kg oats DM/day), during pregnancy. All mares foaled uneventfully at term with a mean ± SD gestational length of 343 ± 11.1 days. Foals were bottle fed with 1.2 L of colostrum in divided feedings before 4 hrs post foaling, unless they were found already nursing and they were then bottle fed 600 mL of colostrum. Table 4.1 shows the manufacturer's guaranteed analysis of the vitamin and mineral content of the commercial granulated equine vitamin and mineral supplement (Masterfeeds).

Mare and foal (n=10) jugular blood samples (10 mL) were taken on day 0 (foaling date), and 10 days later using sterile tubes without anticoagulant. Regarding day 0 foal blood samples, all blood samples were taken after the foal received colostrum or was found nursing; six out of ten foals' serum samples were obtained within one-hour post-foaling, whereas, the remaining blood samples were obtained at approximately 4 hrs after birth. Serum samples were refrigerated overnight, centrifuged, serum removed and frozen at -20ºC. Colostrum samples were obtained between one to four hours after birth and were tested for quality using a Brix sugar refractometer (Animal Reproduction Systems, Inc, Chino, CA, USA), the reference ranges used were: 10-15 % Brix: 0-28 g/L IgG content or poor quality; 15-20 % Brix: 28-50 g/L IgG content or borderline quality; 20-30 % Brix: 50-80 g/L IgG content or adequate quality, and >30 % Brix: >80 g/L IgG content or very good quality. Milk samples were obtained by milking one side of the udder while the foal was suckling the other side at 10 days post-foaling. Colostrum and milk samples were stored in 50 mL plastic tubes and frozen at -20ºC until analysis.

4.3.2 Serum, colostrum and milk iodine

Serum from mares (day 0) and foals (day 0 and 10) plus 30 mL of colostrum (day 0) and milk were submitted to the Animal Health Diagnostic Laboratory at University of Guelph, Guelph, ON, for iodine analysis using inductively coupled plasma mass spectrometry (ICP-MS). The detection limit of ICP-MS method for serum, colostrum and milk iodine was 4 µg/L. The reference ranges used were according to Puls (1994) [41] and Mochizuki et al. (2016) [8]. The adult horse serum iodine ranges were: <10 µg/L deficient, 11- 20 µg/L marginal, 21 - 49 µg/L adequate, >50 µg/L high, and for milk iodine were: <4 µg/L deficient, 10 – 16 µg/L adequate, and 50 µg/L toxic [41].

4.3.3 Thyroid hormones

Basal thyroid hormone levels of total thyroxine (TT4), and total tri-iodothyronine (TT3) from mares and foals were determined at day 0 and day 10 using the Siemen's Immulite TT4 and TT3 kits (Siemens Immulite Canine TT4, and TT3, 1577 North Service Road East, Oakville, Ontario, Canada L6H 0H6) validated for use with equine serum at the Endocrine Laboratory, Prairie Diagnostic Services, Saskatoon, SK. The analytical sensitivity of the TT4 assay was 0.15

nmol/L. Specificity data regarding the anti-TT4 antibody from the manufacturer identified 100% cross-reactivity with L-thyroxine, 55% with D-thyroxine, 16% with tetraiodothyroacetic acid, and 3.2% with triiodo-L-thyronine. The intra-assay coefficients of variation for TT4 were 10.8%, 11%, 13.4% and 8.8% for reference sera with a mean TT4 concentration of 63.9, 119, 10.8 and 21.8 mmol/L, respectively. The analytical sensitivity of the TT3 Immulite assay is 0.54 nmol/L. Specificity data from the manufacturer regarding the anti-TT3 antibody identified 100% cross-reaction with triiodo-L-thyronine, 100% with triodo-D-thyronine, 1.3% with tetraiodothyroacetic acid, and 0.7% with triiodothyroacetic acid. The inter-assay coefficients of TT3 were 7.6%, 6.3%, 9.1%, and 3.2% for reference sera with mean TT3 concentrations of 0.7, 1.1, 3.3, 5.8 nmol/L, respectively. Laboratory personnel were blinded to the identity of the samples. Reference ranges for thyroid hormones in adult horses were obtained from the Animal Health Diagnostic Center Laboratory at Cornell University, Ithaca, NY, 148523. The lower and upper end of the reference range for TT4 is 12.87 - 38.6 nmol/L and for TT3 is 0.46 - 1.088 nmol/L. The mare and foal TT4/TT3 ratios were calculated.

4.3.4 General Data Analysis

All data were analyzed for significance at $P<0.05$. Proprietary software (SPSS version 24a, IBM, 1 New Orchard Road, Armonk, New York, USA 10504-1722) was used for statistical analyses. The data were evaluated for normality and Spearman rank correlations were performed between foal and mare parameters at day 0 and 10, and between foal serum iodine and thyroid hormone levels. Wilcoxon signed rank test was used to determine the difference in thyroid hormones, serum, milk and colostrum iodine concentrations between mares and foals on the day of foaling, and from day 0 to day 10 in foals.

4.4 Results

One foal died of an unknown cause at 10 days of age, and therefore no day 10 serum sample was obtained. Serum from one mare was lost in shipment. Based on reference ranges according to Geor (2013), the mares consuming 2.5% of their body weight in kilograms DM forage, and 0.73 kg oats per day and assuming 10% feed wastage, the iodine content of the base

ration (timothy grass hay plus oats) was estimated at 3.1 mg iodine per day [160]. The iodized salt, vitamin mineral supplement, and cobalt salt intake based on the amount fed and the manufacturer's guaranteed analysis was estimated to provide an additional 35.8 mg iodine per mare per day, for a total of 39 mg iodine or 2.2 mg iodine / kg DM. It was noted that there was variable intake among mares, as some mares were more frequently seen consuming the vitamin/mineral/salt mixture.

4.4.1 Colostrum quality

Based on the sugar refractometer (Brix % type): 5/10 colostrum samples had adequate colostrum quality (20-30 % Brix or 50-80 g/L of IgG content)**,** 3/10 colostrum samples had borderline colostrum quality (15-20 % Brix or 28-50 g/L of IgG content) and 2 samples were not analysed due to laboratory oversight.

4.4.2 Serum, colostrum, milk iodine

Foal serum iodine values are shown in Table 4.2. Foal iodine concentrations were 14.1 times higher (P =.001) than mare levels at birth; and median foal serum iodine levels decreased (P =.012) 3.8-fold from birth to day 10. Colostrum iodine levels were 3.4-fold higher (P =.007) than day 10 milk levels [48 ± 5.6 (31.7, 62)]. Foal serum iodine at foaling date (day 0) and day 10 showed a strong positive correlation with basal TT4 concentrations (r = 0.930, P =.001; r = 0.952, P =.001, respectively). Figure 4.1 shows the relationship between colostrum and milk values for each mare. Figure 4.2 shows the change in each foal's serum iodine level from day 0 to day 10.

4.4.3 Thyroid Hormones

Foal TT4 levels are shown in Table 4.2. Foal serum TT4 concentrations were 65.9-fold higher (P =.005) than mare levels on day 0, whereas, foal TT3 levels were 14.5 fold higher (P =.005) than mare TT3 levels on day 0. In addition, foal TT4 decreased (P =.008) 17.6-fold from day 0 to day 10. Foal TT3 levels decreased (P =.011) 2.6-fold from 0 to day 10. Mare colostrum,

milk and serum iodine and TT4 and TT3 data are also summarized in Table 4.2. Figure 4.3 shows the change in foal TT4 from day 0 to day 10.

4.4.4 TT4/TT3 Ratio

The TT4/TT3 ratio was calculated and data are presented in Table 4.2. At day 0 foal TT4/TT3 ratio (85.9:1) was higher (P =.005) than the mare ratio (16.1). The foal day 10 TT4/TT3 ratio (15.4:1) was lower and different (P =.008) than at day 0. The foal median day 10 TT4/TT3 ratio (15.4:1) was not different from the day 0 mare TT4/TT3 ratio (16.1:1).

4.5 Discussion

Iodine deficiency is one of the top three most prevalent nutritional deficiencies worldwide [8]. Iodine is an essential nutrient needed for thyroid function, growth and metabolism [8,24]. The relationship between iodine status and thyroid function is not well described in horses, as direct investigation into iodine concentrations in horses was previously impossible due to a lack of laboratory capacity [8]. The central role of thyroid function and the long-term consequences on processes such as epigenetic programming have been described in pregnant mares [1].

This is the first paper that describes the rapid change in serum iodine levels in foals after birth. The foal serum iodine levels were 14.1-fold higher than mare levels indicating active transplacental transfer from the mare during late pregnancy. The observed median levels of serum iodine in foals at day 0 were very high 268.5 ± 8.6 µg/L and then decreased over time, reaching a median of 70 ± 23 µg/L ten days later. It is worth mentioning that the high iodine level in foal's serum at foaling day reported in the present study may reflect some intestinal absorption as all blood samples were taken after the foals were bottle fed with colostrum, nevertheless, iodine levels in colostrum samples had a wide variability, ranging from 35 to 383 µg/L. The foal iodine levels were greater than 200 µg/L, with the exception of one foal with 179 µg/L, despite the lower iodine concentrations in their respective mare's colostrum. Iodine transfer through the mammary gland into colostrum is significant and may have caused an increase in serum iodine levels in foals, but the fact that the thyroid hormone levels are very high at birth reflects that the foal received transplacental iodine before parturition. Austin et al (1980) demonstrated that iodine levels in pre sucking calves were high suggesting transplacental transfer rather than ingestion of colostrum as the main source of iodine [40]. Figure 4.2 illustrates the

rapid fall and individual variability in the serum iodine levels from day 0 to day 10. The high iodine levels in the neonatal period mirrored the high foal serum TT4 and TT3 levels, which are shown in Figure 4.3. This is consistent with the rapid fetal growth and final organ maturation and metabolic capacity needed for extra uterine life. Foal serum iodine concentrations were highly correlated with TT4 levels at both time points, and this outcome was expected as TT4 is the main source of organic iodine [24]. Our results in foals are similar to Austin et al. (1980) who studied neonatal dairy calves. They reported the serum iodine levels in one-day old calves from cows receiving two different levels of dietary iodine (0.6 µg and 4.6 µg / kg DM, respectively). Calves from cows fed the lower iodine diet had an average mean serum iodine level of 214 ± 16 µg/L, whereas, calves from the high iodine diet had an average of 960 ± 87 µg/L iodine. Both groups of calves had a marked decline in serum iodine levels over an eight-day period [1,40], showing that iodine supplementation during pregnancy had a direct effect on the calf's serum iodine levels, and that high serum iodine levels are present in apparently healthy growing calves. Both healthy calves and foals have high iodine levels that decrease over time in the neonatal period compared to their dams [1,40].

There is very little published information on serum iodine levels in mares. The observed median ± SE serum iodine levels in mares (19 µg/L ± 3.4) were similar to the results of Japanese investigators who reported lower mean serum iodine (17.5 µg/L) levels in horses in an environment with low soil iodine compared to values from horses in other areas of the country with high soil iodine (22.5 µg/L), or where horses in a high soil iodine region had been supplemented with seaweed (29.33 µg/L) [8].

Using the serum iodine reference ranges from Puls (1994), six out of the ten mares in this study were classified as marginal in serum iodine [41]. Our estimated iodine intake was based on feed iodine values reported by Geor et al., (2013) for forage and grain, and the information from the mare owner on the quantities of mineral fed [160]. We reported under the circumstances of this study that the mare's total dietary intake, which was estimated as 39 mg, of which 35.8 mg came from supplements, was insufficient for the demands of pregnancy based on the serum iodine levels in the mares. The investigators relied on information supplied from the owner, who had a regular schedule for feeding, and who knew the weights of the mineral quantities fed. It is still possible that there is some error introduced to this study because of this fact, and because the

intake of the mares was voluntary and not controlled, and the amount of the mineral that may have been wasted could not be directly accounted for. The recommended range of iodine for mares has been reported to be between five to ten micrograms per kilogram body weight [161]. The calculation for iodine using this range would yield from 3.4 mg – 6.8 mg total daily iodine intake required for the draft mares in this study. There was no evidence of iodine toxicity, such as goiter formation, in the mares and foals in this study [8]. Based on the total amount of iodine (mg/kg) provided the mares received 7.2 times more than the recommended daily intake by the National Research Council for a 680 kg horse during pregnancy, (5.44 mg of iodine per day) [161] and 5.7 times more than the upper iodine intake recommended (6.8 mg per day) [161]. All mares in this study foaled apparently healthy foals and iodine levels in colostrum and milk were considered adequate or higher based on Puls (1994), suggesting that despite marginal levels of iodine in serum and high mineral supplementation levels, maternal iodine transfer to the fetus was adequate during pregnancy and early lactation [41]. Puls (1994) has no reference levels listed for colostrum and reports milk iodine levels > 50 µg/L as toxic. The high levels we measured are likely within the normal physiologic range for milk and caution should be exercised when interpreting iodine levels in the early post-partum period. The higher end of the reference range for milk or colostrum iodine may require adjustment.

The mares in this study had access to a loose salt / mineral mix and a salt block. Schryver et al. (1987) reported a wide range of voluntary daily salt intake from salt blocks, with the upper limit being of 0.35 g salt per kilogram body weight without having detrimental effects on the horse's health [162]. These authors reported a mean voluntary intake of 55 g per day from salt blocks for 400 – 550 kg horses. Applying the 0.35 g/kg salt consumption per day for a 680 kg mare, the maximum voluntary consumption would be 238 grams of salt per day. Other authors however reported a range of 14 – 19 g of block salt per day from salt blocks in athletic horses, with consumption of salt affected by feeding frequency [76]. In the present study based on calculations using the amount of block salt fed the mares would have on the average consumed 77 g of block salt per day, 9.2 g of salt in the mineral mix and 115.5 g of the iodized salt which totals 201.7 g, which is close to the 0.35 g/kg salt voluntary intake reported (238 g). The combination of the amount of loose salt mixed with the palatable vitamin and mineral

supplement may have resulted in a relatively high intake per day per mare and thus a high iodine intake because horses will consume more loose salt than salt from a block [68].

Based on mare's serum iodine data it is possible that pregnant mares under these conditions have a higher demand for iodine than has been reported [162]. In many species, iodine requirements double during pregnancy. The marginal serum iodine levels in the group may be explained by individual mares not consuming sufficient amounts of the supplements, excessive amounts of wastage, high excretion rates, or other dietary components which may have influenced iodine uptake [66]. Other dietary factors that may impact serum and milk iodine levels include the ingestion of feeds containing goitrogenic compounds including wild or cultivated plants in the *Brassica* family such as canola, rapeseed, and mustard, or compounds such as nitrates in feed or water [66]. These dietary factors would have to be considered when interpreting serum and milk levels of iodine in most species, and no attempt was made to control for these in this study [66].

In the present study, the median iodine level in mare colostrum was 165 µg/L with a minimum and maximum range of 24 and 383 µg/L, respectively. Figure 1 shows a decrease by day 10 in all mares to approximately half of the initial value, with a median value in mare milk of 48 µg/L and a minimum and maximum range of 15 µg/L and 153 µg/L respectively, at 10 days post foaling. This is similar to the data reported by Navrátilová et al. (2019) concerning iodine levels in mare's milk in the first month of lactation as 120 µg/L [42]. We concluded that the mare's mammary tissue was able to concentrate iodine in colostrum and milk and this would be a source of iodine for foals. We note that milk production is variable among mares, which can contribute significantly to the variation seen in milk iodine levels. Other authors have reported a decrease in other mineral levels during the transition from equine colostrum to milk [163,164]. Navrátilová et al. (2019) report that mare milk iodine level decline in the first 6 months post-partum from 120 µg/L to 28 µg/L [42]. Kavazis et al. (2002) and Csapo-Kiss et al. (1995) reported higher copper (Cu) and zinc (Zn) levels in colostrum compared to milk at 28 days post foaling, with an average of 1,110 µg/L versus 350 µg/L for Cu and 2,880 µg/L versus 174 µg/L for Zn [163,164]. Similarly, Fantuz et al. (2013) reported a mean level of iodine, Zn and Cu in donkey milk samples taken from 46 to 142 days post foaling of 74.9 µg/L, 2,246.8 µg/L and 97.6 µg/L, respectively, and that concentrations of iodine and Zn were lower in serum compared to

milk, suggesting that the mammary tissue of jennies was regulating the transfer and concentration of these trace minerals into milk [46]. The data from the jennies is similar to the pattern observed in the present study with mares, in which colostrum samples had higher levels of iodine compared to serum samples, and milk [46]. The jennies had higher milk iodine levels than the mares in this study [46]. This may be attributed to the level of iodine in the environment and the rate of supplementation in the diet. In dairy cows a 75% increase in iodine supplementation from 0.5 mg/kg to 2.0 mg/kg of diet DM increased iodine levels in milk within 22 hours, going from an average of 416 to 602 µg/L; a 45% increase [48], whereas an 80% increase in iodine supplementation from 0.82 mg/kg to 4.0 mg/kg in jennies resulted in an 25% increase in iodine levels in milk compared to the control group whose iodine intake was 0.82 mg/kg [46]. This data indicates in equids the mammary gland is able to respond efficiently to dietary variations in iodine during late pregnancy and lactation similar to dairy cows. The dietary level of iodine (4 mg/kg) in the study of Fantuz et al. (2013) was higher than the amount of iodine (2.4 mg/kg DM) estimated to be consumed by the mares in the present study [46].

Thyroid hormones and iodine are essential for fetal maturation before parturition and for neonatal adaptation [11,24]. Dramatic pre-partum increases in cortisol and TT3 in fetal circulation are reported to be essential for extrauterine survival, such as lung maturation, thermogenesis, hepatic gluconeogenesis and nervous system development [11,24,121]. Similar to other authors we report high levels of TT4 and TT3 in the post-natal period [24]. The median serum TT4 level in the foal was 65.9 times higher than the mare level on day 0. Both TT4 and TT3 rapidly declined from day 0 to day 10 in the foals, with a proportionately greater decline in TT4 (17.6-fold) than TT3 (2.6-fold). We report a positive correlation with foal serum iodine and TT4 in healthy foals. It has been reported that deficiencies in thyroid function caused by either excessive or inadequate amounts of iodine or due to exposure to goitrogens during the critical phases of fetal growth and maturation, have serious negative consequences for the foal, and have adverse effects on the foal's future health and performance [24,165]. Imbalances in dietary iodine have been reported to cause goiter in foal [108,166–168]. We anticipate that these adverse maternal and fetal exposures to iodine would alter foal serum thyroid hormone concentrations, serum iodine levels, colostrum and milk iodine levels and the foal TT4/TT3 ratio, but this requires further study.

Table 4.1. Mineral and vitamin composition of the vitamin and mineral supplement.

Mineral and vitamin composition of loose horse mineral supplementation (20 kg)			
Ingredient	**Amount per kilogram**	**Ingredient**	**Amount per kilogram**
Calcium	12.0%	Vitamin A	600,000 IU/kg
Phosphorus	8.0%	Vitamin D3	40,000 IU/kg
Magnesium	1.6%	Vitamin E	2,000 IU/kg
Salt	8.0%	Vitamin K	15 mg/kg
Sodium	3.2%	Vitamin B12	200 mcg/kg
Potassium	4.0%	Biotin	40 mg/kg
Iodine	40 mg/kg	Riboflavin	400 mg/kg
Iron	40 mg/kg	Thiamine	350 mg/kg
Copper	700 mg/kg	Niacin	1,600 mg/kg
Manganese	1,900 mg/kg	Pantothenate	200 mg/kg
Zinc	4,000 mg/kg	Pyridoxine	20 mg/kg
Cobalt	27 mg/kg	Folic Acid	58 mg/kg
Fluorine	1000 mg/kg	Choline Chloride	1,000 mg/kg
Selenium	15 mg/kg		

Table 4.2. Mare and or foal basal serum levels of TT4, TT3 (nmol/L) and iodine µg/L [median ± median standard error (quartiles)] at the day of foaling (day 0) and day 10 post-foaling. Iodine levels µg/L in colostrum at day 0 and milk at day 10 [median ± median standard error (quartiles)] post-foaling.

TT4 nmol/L	Day 0	Day 10
Foal	1225 ± 47.8 (757.7, 1292.7)	69.6 ± 20.4 (60, 98.1)
Mare	18.6 ± 0.6 (15, 21.7)	
TT3 nmol/L		
Foal	14.2 ± 1.1 (11, 21)	5.4 ± 0.3 (4.6, 5.9)
Mare	0.98 ± 0.04 (0.90, 1.40)	
TT3/TT4 ratio		
Foal	85.9 ± 8.5 (46.9, 103.2)	15.4 ± 1.6 (13.4, 16.5)
Mare	16.1 ± 0.6 (14.4, 20.1)	
Serum Iodine µg/L		
Foal	268.5 ± 7.5 (258,293.5)	70 ± 3.6 (54.7, 87.5)
Mare	19 ± 0.5 (17, 22)	
Colostrum Iodine µg/L	165 ± 15.1 (73.5, 238.5)	
Milk Iodine µg/L		48 ± 5.5 (31.7, 62)

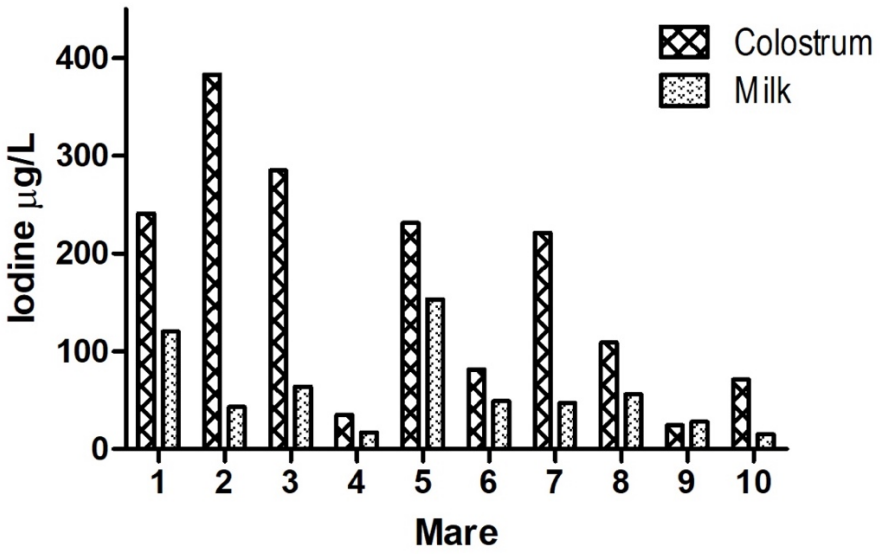

Figure 4.1. Iodine levels (µg/L) in paired colostrum and milk samples at foaling (day 0) and day 10 post-foaling in ten mares.

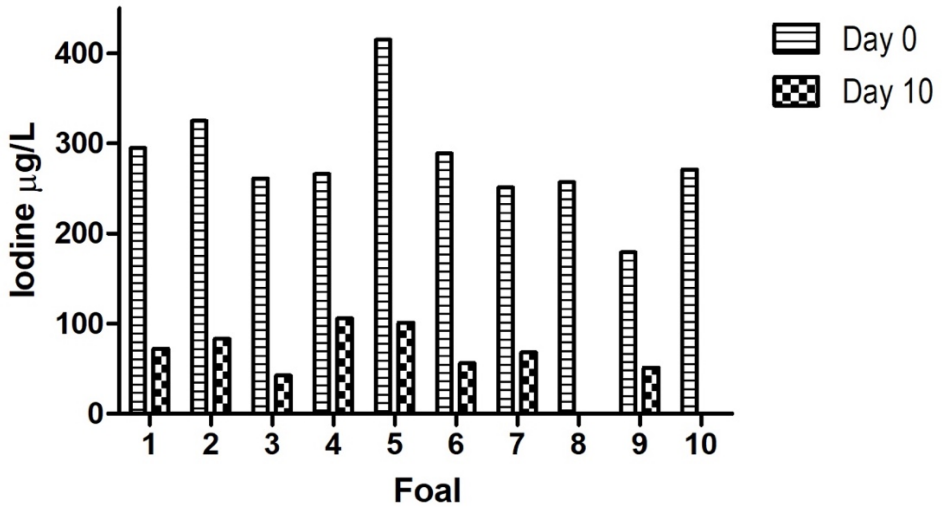

Figure 4.2. Serum iodine levels (µg/L) in paired samples obtained from ten foals on the day of birth (day 0) and day 10 post-foaling.

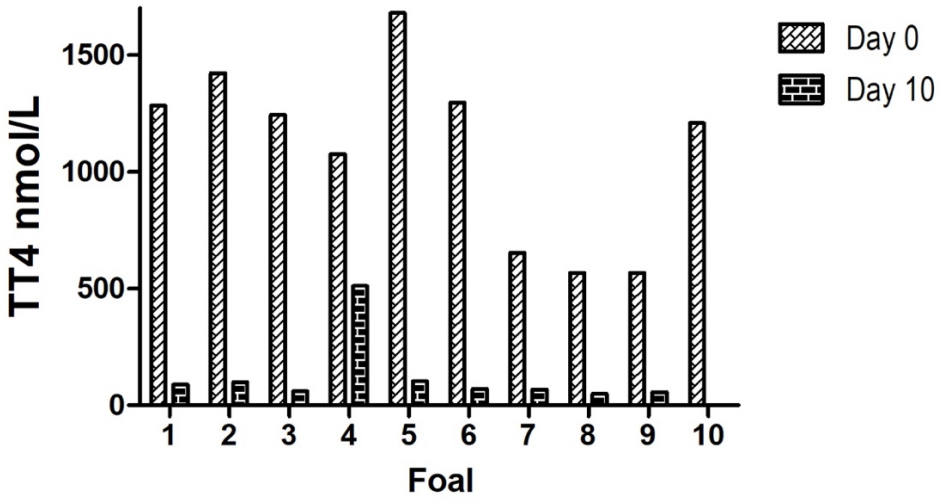

Figure 4.3. Serum total thyroxine levels (TT4) (nmol/L) in paired samples from ten foals on the day of birth (day 0) and day 10 post-foaling.

CHAPTER 5: GENERAL DISCUSSION

The negative effects of GSL on thyroid hormone levels and iodine metabolism have been described in dairy cows and other domestic species, in which adequate levels of iodine in milk products need to be present to meet the requirements for human consumption [47, 66, 83]. Nevertheless, to the author's knowledge there are no previous reports on the effect of glucosinolates on thyroid function and iodine levels in horses at any age and physiologic state; there is also a lack of robust information on normal iodine levels in mares and foals in different body fluids and secretions despite the prevalence of congenital hypothyroidism dysmaturity syndrome (CHDS) in foals in Western Canada, where *Brassica* family plants are widely cultivated, mustard family weeds are abundant and iodine concentration in the soil is inadequate [65, 134, 135, 140]. Iodine and thyroid hormone levels are linked as iodine is necessary for thyroid hormone synthesis [51, 65]. Adequate levels of thyroid hormone concentrations are essential for normal fetal development, especially growth and maturation of the musculoskeletal system in the second and third trimester of gestation [59]. Maternal thyroid hormones are reported to be unable to reach the fetal circulation from the second trimester of pregnancy to term in species with an epitheliochorial placenta such as the horse and ruminants, but the fetal hypothalamic-hypophyseal-thyroid axis is active by this stage and stimulates the synthesis of fetal thyroid hormones [11, 55]. Nevertheless, iodine transfer from maternal to fetal circulation is essential for normal fetal thyroid hormone synthesis but also for adequate gas exchange, thermogenesis and hepatic gluconeogenesis in the neonatal period [11]. In order to maintain adequate fetal thyroid hormone synthesis, iodine transfer from maternal to fetal circulation is essential, thus, maternal iodine deficiency caused by nutritional deficiency and/or goitrogenic compounds that compete with iodine transport into the thyroid gland can lead to thyroid dysfunction in the fetus [40, 44]. In infants, it is well documented that hypothyroidism during pregnancy severely also affects brain and cognitive development of infants [28].

A syndrome characterized by prolonged gestational length, moderate to severe musculoskeletal deformities such as contracted tendons, failure of tarsal and/or carpal ossification, prognathia, signs of dysmaturity and goiter called congenital hypothyroidism dysmaturity syndrome in foals (CHDS) has been described in Western Canada since the late 1970's and investigated by Allen and coworkers. All of the risk factors associated with the

presentation of CHDS and the pathogenesis is not completely understood but lack of trace mineral supplementation, feeding green feed, and consumption of plants containing GSL during pregnancy have been reported. Glucosinolates (GSL), which are commonly found in *Brassicaceae* family plants, have been reported to have negative effects on animal health. Major GSL metabolites such as isothiocyanates, thiocyanates and oxazolidinethione disrupt iodine availability to the thyroid, thus causing thyroid dysfunction. The effect of GSL has been described in other domestic animals but there is little information in horses. Research in dairy cows receiving rapeseed meal for example, showed that the cow's iodide concentrations in milk decreased while the levels of thiocyanate increased.

We aimed to characterize iodine levels in serum, colostrum and milk samples along with thyroid hormone concentrations from mare and foal pairs at foaling date and ten days later, as well as the effects of the GSL sinigrin in combination with a low iodine supplemented diet on thyroid hormone and iodine concentrations in non-pregnant mares. The idea was to determine the normal iodine reference range in mares and foals from different body fluids and recreate the conditions that were reported to be risk factors for CHDS in foals to determine if there is a negative effect on thyroid hormone concentrations and iodine levels in blood from mares exposed to the mustard meal.

In the first study (chapter 3), we partially accepted our hypothesis that the combination of sinigrin and a low iodine diet altered thyroid hormone concentrations. It was clear that serum iodine increased in the Control group mares and decreased in the GSL mare groups by week 12. The experimental design did not allow us to clearly determine which factor (sinigrin, or low iodine) or if both were responsible for the thyroid response to treatment. The study was started in late summer and concluded in late fall just before the winter solstice. The weather was extremely cold in the month of November during the trial and at this latitude daylight was decreasing rapidly. The data showed a marked response to TRH in terms of thyroid hormone secretion in the Control mares and this effect was absent in the GSL mares. At week 12 the response to the TRH was similar in the GSL mares to their week zero responses. The question was whether the marked response to TRH in the Control group mares was due to a seasonal effect. As there were no studies performed under similar management conditions, or with similar climatic and geographic factors, it was unclear whether the enhanced response to TRH in the Control group mares was due to the iodine supplemented diet, a seasonal or climatic effect, the combination of

diet with a seasonal effect, or conversely whether there was a suppression of the response to TRH in the GSL groups mares at week 12. The cause of the suppression in the TRH response in the GSL groups, if it was present, may be related to the effects of the GSL diet, such as through the depletion of stored iodine in the thyroid or other factors. It was apparent from many studies that the effects of GSL may be diminished by supplementation with iodine [48,79,138,150,169–171]. To the authors' knowledge, the effect of iodine supplementation on thyroid responsiveness to TRH in horses has not been addressed; however, studies performed in euthyroid human patients stated that there is a normal transient effect of a high level of iodine supplementation in the diet, in which TT4 concentrations decrease, with a mild increase in TSH levels [157]. This is not compatible with our findings as Control group mares did not show a decrease in basal TT4, and TSH levels were within normal limits. The lack of change in TSH was also of interest because the TSH levels were similar between Control and GSL groups in terms of response to TRH administration, however the thyroid response in terms of the post – TRH release of thyroid hormone and conversion from T4 to T3 were different in Control versus GSL groups. The response to TRH is not fully dependent on the thyroid hormone stores within the gland [19]. In the mare it has not been determined what portion of the TRH response is due to the release of the stored hormone in the thyroid gland and what proportion is due to the effects of TSH on the changes in peripheral conversion of T4 to T3 [172,173]. The post TRH peak level of T3 occurs at 2 hours, while the peak in T4 occurs at 4 hours [174]. The effect on the thyroid response to TRH could be explained by: changes in thyroid hormone receptor expression or binding; effects of GSL on iodide transport and depletion of thyroid hormone stores; changes in peripheral conversion by deiodinases; or other factors. Some studies in people have reported that thyroid health may be affected by GSL and dietary iodine without altering basal TSH levels [85,175]. Further studies need to be performed to determine why the response to TRH in terms of thyroid hormone concentrations was different while TSH levels were similar in the experimental groups.

Regarding serum iodine levels, there was a noticeable increase in the Control group, whereas GSL groups decreased their levels by week 12 compared to week 0. The decrease in serum iodine would be an expected outcome of a 12-week dietary sinigrin exposure. It was our opinion that the change in serum iodine was unlikely to be caused by the low iodine diet alone; however, the experimental design does not allow us to draw a conclusion in this regard. Because the goal of the study was to recreate the reported risk factors for CHDS, which included a lack of

trace mineral supplementation and exposure to possible goitrogens, we avoided supplementation with additional iodine in the GSL groups [6,176]. Eisenbrand and Gelbke (2016) reported that sodium iodide symporter inhibitors may decrease thyroid iodine by 20% [99]. It has been reported that supplementation with iodine has been shown to counteract the effects of GSL [50]. It is worth mentioning that the lack of a GSL group with iodine supplementation, or a control group without iodine supplementation along with the measurement of iodine and creatinine levels in urine would have strengthened the study. Without this information there are still knowledge gaps in the understanding of the effect of GSL on iodine levels in horses. This was an unavoidable consequence of the financial limitations of the study. Furthermore, there is little information regarding the tolerance and palatability of sinigrin, which is metabolized to isothiocyanates, and the negative effects may have been underestimated as the oriental mustard powder is intended to be used for human consumption, thus, GSL detoxification may have been performed, or the processing method may have decreased the GSL content.

It was beyond the scope of this study to measure the metabolites of sinigrin in the mare's serum. Based on the present study, a concentration of 20 – 35 mmol/day of sinigrin was adequately tolerated by the Low and High GSL groups, respectively, until the end of the feeding trial and there was no change in feed consumption. We note that not all mares would consume the mustard as horses are selective eaters and this prevented full randomization of the mares into groups. Lastly the numbers are relatively small in the study and the data must be viewed with this in mind.

The results from the third chapter indicates that the GSL sinigrin is able to compete with iodine uptake by the thyroid gland in the non-pregnant mare, nevertheless, there is still limited and inconsistent information regarding normal serum and milk iodine levels in adult mares, and there is no reference range reported in foals. Since iodine is an essential trace mineral for thyroid hormone synthesis and the lack of information can interfere with an accurate interpretation of the results, the goal of the second study (chapter 4) was to characterize the serum iodine values in postpartum mare foal pairs, and levels of iodine in milk and colostrum. The results showed clear evidence of active transplacental transfer of iodine from the mare to the fetus during late pregnancy, as iodine levels in serum samples from neonatal foals taken between one to four hours post-foaling revealed a 14.1-fold higher level than the mare. Even though pre suckling samples were not obtained for iodine measurement, studies performed in cattle showed that

iodine concentrations were more highly correlated with levels in the feed than in colostrum [49]. We have evaluated a few samples from foals before and after colostral ingestion and the high serum iodine level is present before suckling (data not shown) in agreement with this finding. As the main source of organic iodine is thyroid hormone the iodine levels in the foals were correlated and consistent with their serum iodine levels.

Furthermore, under the circumstances of this study and based on specific information supplied from the owners, the estimated voluntary iodine intake per day / mare was of 39 mg, of which 35.8 mg came from supplements. What was noteworthy was that this level of iodine supplementation was insufficient for the demands of pregnancy based on the serum iodine levels in the mares, as six out of ten mares had marginal serum iodine levels despite iodine supplementation through the entire pregnancy. A limitation of this field study was that the mineral intake of the individual mares was not controlled and may have been uneven between mares. Nonetheless it is worth mentioning that the recommended range of iodine intake for mares is between five to ten micrograms per kilogram body weight, thus, a calculation using this range would mean that the total daily iodine intake would range from 3.4 mg – 6.8 mg. The mares' intake was therefore much higher than the recommended level however, there was no evidence of iodine toxicity, such as goiter formation, in the mares and foals in this study [177]. This may be interpreted to mean that the demands of late pregnancy for iodine are much higher than the reported NRC recommendations. It would have been optimal if 24-hour urine creatinine and iodine levels could have been assessed to determine if iodine metabolism and excretion was within normal limits. Also, because this was a field study, there was no attempt to control for factors such as antithyroid compounds present in the environment and feed, such as nitrate or GSL. This may explain why iodine intake levels were high, yet 6/10 mares had marginal serum iodine levels as these substances interfere with iodine uptake and iodine is excreted in the urine. While the mares in this study showed no evidence of toxicity, it has been reported that iodine levels that induce toxicity are often in excess of 10 times higher than the recommended dietary level [177]. In humans it has been reported that women in Japan may have 10 - 25 times the recommended amount of iodine in their diet, as it is customary to consume seaweed which is an iodine rich food, and do not show signs of toxicity [178]. It may be that different species adopt to their environmental circumstances of iodine excess or deficiency, or that the range of tolerance is much higher before iodine toxicity is induced [27,170].

On the other hand, Puls (1994) has no reference levels listed for iodine concentrations in colostrum and milk iodine levels > 50 µg/L are reported to be toxic [41]. We concluded that the recommended levels of iodine in milk do not apply to colostrum, as colostrum values are significantly higher. Colostrum and milk both have higher iodine levels than serum showing that the mammary tissue of the mare is able to concentrate iodide. Navrátilová et al. (2019) provided valuable information on the iodine level in milk by month of lactation [42]. The high levels of iodine we reported in colostrum and milk samples were from mares that showed no evidence of iodine toxicity, suggesting that the higher end of the reference range for milk iodine may require adjustment, and a separate range should be provided for colostrum. The mare apparently makes a significant investment in the health of her fetus and neonate by supplying her offspring with minerals [163]. We concluded that further studies should be performed in mares with known and confirmed iodine intakes to create more robust reference ranges for serum, colostrum and milk.

Iodine is essential for thyroid hormone formation. There is wide recognition of the role of thyroid hormones in epigenetic programming in horses [126,179]. Important processes such as metabolism, boney development, are controlled by thyroid hormones [180,181]. Thyroid hormones and iodine concentrations in foal serum were highest on the foaling date and decreased over a ten-day period of time. There was a positive correlation between foal serum iodine and TT4 in healthy foals, indicating the important role of iodine in the highly active thyroid gland during the neonatal period. This high level of thyroid hormone has been reported to be critical for neonatal adaption [11]. This study therefore makes important contributions in establishing the iodine concentrations in foals and their relationship to thyroid hormone levels.

CHAPTER 6: CONCLUSIONS AND FUTURE STUDIES

The long term objective of this research was to have a better understanding of the impacts of a low iodine supplemented diet combined with GSL exposure in non-pregnant mares with a view towards utilizing the data for a subsequent study in pregnant mares, where the impact on fetal and neonatal development and maturation would be examined. Low dietary iodine and goitrogenic GSL are considered to be risk factors for mares that deliver foals with congenital hypothyroidism dysmaturity syndrome. Goitrogenic GSL have been confirmed to affect thyroid function and fetal musculoskeletal development in other farm animals; however, the effect of GSL in combination with inadequate mineral intake, such as iodine, in horses is unknown. There is generally a reluctance to fund studies that propose to euthanize horses and foals and this limits the capacity to evaluate detailed changes in musculoskeletal tissues or thyroid iodine content.

Sinigrin is a GSL that is not as highly goitrogenic as other GSL [66,132,182]. The metabolites of singrin such as nitriles and allyl-isothiocyantes are less potent than other GSL whose metabolites include isothiocyanates (progoitrin) [169,183–185]. Sinigrin was chosen because it was available in a convenient oriental mustard powder. The production of a crushed mustard seed meal for the study was logistically problematic because of the many regulatory barriers to having a GSL containing feed prepared. There are also significant production costs associated with cleaning the crushing and milling equipment to remove the GSL from mustard seeds.

We found the combination of low dietary iodine and sinigrin reduced serum iodine levels over time in the mares. There is much left to discover about the effects of GSL in horses. Cymbaluk (1991) and Oliviera et al. (2001) reported no deleterious effects of feeding canola meal to growing horses for a few months and the mares in our study did not show adverse health outcomes [141,142]. The sensitivity of the pregnant mare and her fetus to GSL remains to be determined. As there was a lack of laboratory capacity in measuring iodine for a prolonged period there is a gap in our understanding regarding the effects of GSL in horses and their interactions with other minerals such as selenium and substances such as nitrates and nitrites. In addition, the authors note that robust reference limits for serum, colostrum and milk iodine in horses are lacking. For that reason, reference values for iodine concentrations in mare and foal serum, as well as in colostrum and milk samples need to be further examined in order to

diagnose and study problems such as iodine deficiency disorders in the future. This thesis constitutes a first approach to these goals.

Based on the results of the presented studies we concluded that:

- Based on the palatability trial, there was a wide variability in the willingness of the mares to consume the mustard meal oat mixture; approximately 24% of the mares fully tolerated the bitter taste of the sinigrin, and another 24% partially tolerated the taste, suggesting that under natural conditions some mares may consume more than others of this type of GSL. Mares that consume this type of GSL would be more exposed to goitrogens than other mares.
- The effect of feeding a diet containing sinigrin at a concentration of 20 – 35 mmol per day in combination with a low iodine supplemented diet to non-pregnant mares decreased serum iodine concentrations by week 12, based on the significant decrease in iodine concentrations in the GSL groups. This was more evident in the High GSL group compared to the Control group. The GSL sinigrin, whose secondary metabolite includes allyl-isothiocyanate, may have prevented iodine uptake by the thyroid gland. Consumption of sinigrin would be expected to result in increased renal excretion of iodine resulting in lower iodine serum concentrations over time.
- Feeding sinigrin for a 12-week period impaired TT4 TRH responses by week 12, with no effect on TT3 and TSH response in the GSL groups. Therefore, primary hypothyroidism was not present, as the TSH response was adequate between groups. Impaired and/or low TT4 TRH response has been reported in early stages of hypothyroidism in dogs, suggesting that in the present study, a three-month trial was not long enough to cause more pronounced effects on THs levels.
- The effect of sinigrin along with a low iodine supplemented diet did not have a negative impact on body weight and overall health. Nevertheless, the study was conducted in non-pregnant adult mares, in which thyroid function is less critical compared to thyroid function in pregnant mares or a developing fetus.
- Neonatal foals have higher serum iodine levels than mares, and serum iodine is positively correlated with foal TT4 concentrations in the first week of life.
- Foal serum iodine, TT4 and TT3 concentrations decrease rapidly from day 0 to day 10 in the neonatal period.

- Equine mammary tissue is able to concentrate iodine beyond plasma levels, making colostrum and milk a significant source of iodine for foals, similar to other trace minerals.
- Mare colostrum contains higher amounts of iodine compared to mare milk and may be a source of iodine for foals.
- The estimated voluntary iodine intake of the draft mares exceeded approximately 7 times the NRC recommended iodine intake in the mares in the field study without signs of toxicity. This indicated that the requirements for iodine during pregnancy should be determined using controlled nutritional trials.

The limitations of the presented studies are:

- The group sizes were small in both studies.
- Serum iodine was measured, and urine iodine / creatinine levels were not determined. A comparison between serum and urine iodine concentrations indexed to creatinine are recommended in future studies to have a better understanding of iodine uptake.
- The time of year or the inclusion of iodine supplementation to the control group may have influenced the Control mare's response to TRH. We suggest that lack of TT4 response to TRH in the High GSL group compared to the Control group may have been due to: differences in iodine supplementation, GSL effects, seasonal factors or a combination of these factors. There were unfortunately insufficient research funds to include a Control low iodine group, or a high GSL group with iodine supplementation.
- The field study of serum, colostrum, milk iodine and thyroid hormone concentrations in mares and foals is limited because it is observational data.
- Individual mare mineral/loose iodine mixture intake was not determined, and the hay and oats were not analysed for iodine content.
- The presence of other environmental (example perchlorates) or feed (example nitrates, GSL) antithyroid agents was not investigated in the field study.
- Iodine levels in pre and post-suckle foals were not determined. Therefore, there is still the question of whether high iodine serum levels seen in day 0 foals resulted partially from colostrum ingestion.

Further research is needed to determine:

- The lowest amount of GSL that when fed with a low iodine diet suppresses serum iodine concentrations;
- The effects of various different GSL on thyroid hormone and iodine concentrations and the response to TRH;
- The concentration of dietary iodine required to protect mares from the effects of GSL.
- The effects of a low iodine supplemented diet along with goitrogenic GSL on pregnant mares and their newborn foals.
- A determination if there is a seasonal response to TRH in mares in northern latititudes

In addition, future work is required to better determine reference limits for serum, colostrum and milk iodine in mares with known and confirmed iodine intakes

- The pre suckle and post suckle iodine level in neonates based on observational studies in which serum and milk iodine concentrations of pregnant and lactating mares are examined in light of confirmed iodine intakes.
- Studies comparing 24-hour urine creatinine iodine ratios to spot urine creatinine iodine determinations in horses with known and confirmed iodine intakes to determine if a spot measurement approximates the 24-hour losses.

CHAPTER 7: REFERENCE LIST

[1] Peugnet P, Robles M, Wimel L, Tarrade A, Chavatte-palmer P. Management of the pregnant mare and long-term consequences on the offspring. Theriogenology 2016;86:99–109.

[2] Brinkmann L, Gerken M, Hambly C, Speakman JR, Riek A. Thyroid hormones correlate with field metabolic rate in ponies, equus ferus caballus. J Exp Biol 2016;219:2559–66.

[3] Fitzgerald BP, Davison LA. Thyroxine conentrations are elevated in mares which continue to exhibit estrous cycle during the nonbreeding season. J Equine Vet Sci 1998;18:48–51.

[4] Messer NT, Johnson PJ. Evidence-based literature pertaining to thyroid dysfunction and cushing's syndrome in the horse. Vet Clin Equine 2007;23:329–64.

[5] Mcllwraith C, James L. Limb deformities in foals associated with ingestion of locoweed by mares. J Am Vet Med Assoc 1982;181:255–8.

[6] Allen A, Townsend HGG, Doige CE, Fretz PB. A case-control study of the congenital hypothyroidism and dysmaturity syndrome of foals. Can Vet J 1996;37:349–58.

[7] Swerczek TW. Abortions in thoroughbred mares associated with consumption of bulbosus buttercups (ranunculus bulbosus l). J Am Vet Med Assoc 2016;248:669–72.

[8] Mochizuki M, Hayakawa N, Minowa F, Saito A, Ishioka K, Ueda F, et al. The concentration of iodine in horse serum and its relationship with thyroxin concentration by geological difference. Environ Monit Assess 2016;188:2–6.

[9] Swerczek TW, Dorton AR. Effects of nitrate and pathogenic nanoparticles on reproductive losses , congenital hypothyroidism and musculoskeletal abnormalities in mares and other livestock : new hypotheses. Anim Vet Sci 2019;7:1–11.

[10] Toribio RE, Duckett WM. Thyroid gland. Equine Intern. Med. 2n ed., W.B Saunders; 2004, p. 1340–56.

[11] Forhead AJ, Fowden AL. Thyroid hormones in fetal growth and prepartum maturation. J Endocrinol 2014;221:87–103.

[12] Reed S, Bayly W, Sellon D. Disorders of the endocrine system. Equine Intern. Med. 3rd ed, Saunders; 2010, p. 1251–60.

[13] Schlotthauer C. The incidence and types of diseases of the thyroid gland of adult horses.

JAVMA 1931;78:211–8.

[14] Davies S, Barber D, Crisman M, Tan R, Larson M, Daniel G. Quantitative pertechnetate thyroid scintigraphy and the ultrasonographic appearance of the thyroid gland in clinically normal horses. Vet Radiol Ultrasound 2010;51:674–80.

[15] Viana GF, Carandina L, Hataka A, Midon M, Sarkis CA, Filho JNPP, et al. Ultrasonographic features of the normal thyroid gland in adult horses. Pesq Vet Bras 2019;39:923–31.

[16] Venzke W. Equine endocrinology. Sisson Grossman's Anat. Domest. Anim. 5th ed, Philadelphia: W.B Saunders; 1975, p. 533–50.

[17] Bacha LM, Bacha WJ. Color atlas of veterinary histology. 2nd ed. Lippincott Williams & Wilkins; 2000.

[18] Molina PE. Thyroid gland. Endocr. Physiol. 5e ed., New York, NY: McGraw-Hill Education; 2018.

[19] Breuhaus BA. Disorders of the equine thyroid gland. Vet Clin Equine 2011;27:115–28.

[20] Bassett DJ, Williams GR. Role of thyroid hormones in skeletal development and bone maintenance. Endocr Rev 2016;37:135–87.

[21] Silva J, Ocarino NM, Rogeria S. Thyroid hormones and female reproduction. Biol Reprod 2018;99:907–21.

[22] Zoeller TR, Tan SW, Tyl RW. General background on the hypothalamic-pituitary-thyroid (HPT) axis. J Allergy Clin Immunol 2007;37:11–53. https://doi.org/10.1016/j.jaci.2012.05.050.

[23] Forhead AJ, Curtis K, Kaptein E, Visser TJ, Fowden AL. Developmental control of iodothyronine deiodinases by cortisol in the ovine fetus and placenta near term. Endocrinology 2006;147:5988–94.

[24] Breuhaus BA. Thyroid function and dysfunction in term and premature equine neonates. J Vet Intern Med 2014;28:1301–9.

[25] Breuhaus B. Serum thyroid hormone and thyrotropin concentrations in adult horses as they age. J Equine Vet Sci 2018;68:21–5.

[26] Fazio E, Medica P, Cravana C, Messineo C, Ferlazzo A. Total and free iodothyronine levels of growing thoroughbred foals: effects of weaning and gender. Livest Sci 2007;110:207–13.

[27] Medica P, Fazio E, Cravana C, Ferlazzo A. Influence of endemic goitre areas on thyroid hormones in horses. Animal 2011;5:82–7.

[28] Moog NK, Entringer S, Heim C, Wadhwa PD, Kathmann N, Buss C. Influence of maternal thyroid hormones during gestation on fetal brain development. Neuroscience 2017;342:68–100.

[29] Hartoft-Nielsen ML, Boas M, Bliddal S, Rasmussen AK, Main K, Feldt-Rasmussen U. Do thyroid disrupting chemicals influence foetal development during pregnancy? J Thyroid Res 2011;2011:1–14.

[30] Fazio E, Medica P, Cravana C, Bruschetta G, Ferlazzo A. Seasonal thyroid and lipid profiles in thoroughbred pregnant and nonpregnant mares (equus caballus). Theriogenology 2016;85:1582–9.

[31] Fazio E, Medica P, Trifiletti C, Ferlazzo A. The outcome of the first stages of pregnancy on mares' bloodstream thyroid hormones. Theriogenology 2016;86:1036–41.

[32] Boeta M, Zarco L. Luteogenic and luteotropic effects of eCG during pregnancy in the mare. Anim Reprod Sci 2012;130:57–62.

[33] Johnson A. Serum concentrations of prolactin, thyroxine and triiodothyronine relative to season and the estrous cycle in the mare. J Anim Sci 1986;62:1012–20.

[34] Habibu B, Kawu MU, Aluwong T, Makun HJ. Influence of seasonal changes on physiological variables , haematology and serum thyroid hormones profile in male red sokoto and sahel goats. J Appl Anim Res 2017;45:508–16.

[35] Geor RJ, Harris PA, Coenen M. Equine applied and clinical nutrition: health, welfare and performance. Saunders; 2013.

[36] Leung AM, Pearce EN, Hamilton T, He X, Pino S, Merewood A, et al. Colostrum iodine and perchlorate concentrations in boston-area women: a cross-sectional study. Clin Endocrinol (Oxf) 2009;70:326–30.

[37] Ahad F, Ganie SA. Iodine, iodine metabolism and iodine deficiency disorders revisited. Indian J Endocrinol Metab 2010;14:13–7.

[38] Leung A, Pearce E, Braverman L. Role of iodine in thyroid physiology. Expert Rev Endocrinol Metab 2010;5:593–602.

[39] O'Connor S, Fowden AL, Holdstock N, Guissani D, Forhead AJ. Developmental changes in pulmonary and renal angiotensin-converting anzyme concentrations in fetal and

neonatal horses. Reprod Fertil Dev, vol. 14, 2002, p. 413–7.

[40] Austin A, Whitehead D, Le Du Y, Brownlie J. The influence of dietary iodine on iodine in the blood serum of cows and calves in the perinatal period. Res Vet Sci 1980;28:128–30.

[41] Puls RW. Mineral levels in animal health: Diagnostic Data. . 2nd ed. Clear Brook, B.C. Canada: Sherpa International; 1994.

[42] Navrátilová P, Borkovcová I, Kaniová L, Dluhošová S, Zachovalová H. The content of selected vitamins and iodine in mare's milk. Acta Vet Brno 2019;88:473–80.

[43] Aumont G, Tressol J. Improved routine method for the determination of total iodine in urine and milk. Analyst 1986;111:841–3.

[44] Osame S, Ichijo S. Clinicopathological observations on thoroughbred foals with enlarged thyroid gland. J Vet Med Sci 1994;56:771–2.

[45] Silva C, Merkt H, Bergamo P, Barros S, Barros C, Santos M, et al. Consequence of excess iodine supply in a thoroughbred stud in southern brazil. J Reprod Fert Suppl 1987;35:529–33.

[46] Fantuz F, Ferraro S, Todini L, Mariani P, Piloni R, Salimei E. Essential trace elements in milk and blood serum of lactating donkeys as affected by lactation stage and dietary supplementation with trace elements. Animal 2013;7:1893–9.

[47] Niero G, Franzoi M, Vigolo V, Penasa M, Cassandro M, Boselli C, et al. Validation of a gold standard method for iodine quantification in raw and processed milk, and its variation in different dairy species. J Dairy Sci 2019;102:4808–15.

[48] Weiss WP, Wyatt DJ, Kleinschmit DH, Socha MT. Effect of including canola meal and supplemental iodine in diets of dairy cows on short-term changes in iodine concentrations in milk. J Dairy Sci 2015;98:4841–9.

[49] Guyot H, de Oliveira LA, Ramery E, Beckers JF, Rollin F. Effect of a combined iodine and selenium supplementation on I and Se status of cows and their calves. J Trace Elem Med Biol 2011;25:118–24.

[50] Kursa J, Trávníček J, Rambeck W, Kroupová V, Vítovec J. Goitrogenic effects of extracted rapeseed meal and nitrates in sheep and their progeny. Vet Med (Praha) 2000;45:129–40.

[51] Konečný R, Hasoňová L, Trávníček J, Samková E, Hladký J, Kří'ová Z. Effect of organic selenium and iodine supplementation on selenium and thyroid hormones status of

lactating ewes and lambs. Acta Vet Brno 2015;65:477–87.

[52] Pan Z, Cui T, Chen W, Gao S, Pearce EN, Wang W, et al. Serum iodine concentration in pregnant women and its association with urinary iodine concentration and thyroid function. Clin Endocrinol (Oxf) 2019;90:711–8.

[53] Pearce EN, Leung AM, Blount BC, Bazrafshan HR, He X, Pino S, et al. Breast milk iodine and perchlorate concentrations in lactating boston-area women. J Clin Endocrinol Metab 2007;92:1673–7.

[54] Allen A, Doige CE, Haas SD, Card CE, Fretz PB, Al AET. Concentrations of triiodothyronine and thyroxine in equine fetal serum during gestation. Biol Reprod Mono 1995;1:49–52.

[55] Chan SY, Vasilopoulou E, Kilby MD. The role of the placenta in thyroid hormone delivery to the fetus. Nat Clin Pract Endocrinol Metab 2009;5:45–54.

[56] Burns R, Azizi F, Hedayati M, Mirmiran P, O'Herlihy C, Smyth PPA. Is placental iodine content related to dietary iodine intake? Clin Endocrinol (Oxf) 2011;75:261–4.

[57] Serakides R, Silva JF, Vidigal PN, Galvo DD, Boeloni JN, Nunes PP, et al. Fetal growth restriction in hypothyroidism is associated with changes in proliferative activity, apoptosis and vascularisation of the placenta. Reprod Fertil Dev 2012;24:923–31.

[58] Wilsher S, Ousey J, Allen W. Observations on the placentae of eight thoroughbred foals born with flexural limb deformities. Equine Vet Educ 2013;25:84–95.

[59] Allen A, Fretz PB, Card CE, Doige GE. The effects of partial thyroidectomy on the development of the equine fetus. Equine Vet J 1998;30:53–9.

[60] McLaughlin B, Doige C, McLaughlin P. Thyroid hormone levels in foals with congenital musculoskeletal lesions. Can Vet J 1986;27:264–7.

[61] Kanoh H, Konishi T. Analytical methods for iodine and iodides. In: Kaiho T, editor. Iodine Chem. Appl. 1st ed, John Wiley & Sons; 2015, p. 15–23.

[62] Capen CC. Mechanisms of chemical injury of thyroid gland. Prog Clin Biol Res 1994;387:173–91.

[63] Boosinger TR, Brendemuehl JP, Bransby DL, Wright JC, Kemppainen RJ, Kee DD. Prolonged gestation, decreased triiodothyronine concentration, and thyroid gland histomorphologic features in newborn foals of mares grazing acremonion coenophialum-infected fescue. Am J Vet Res 1995;56:66—69.

[64] Harris PA, Ellis AD, Fradinho MJ, Jansson A, Julliand V, Luthersson N, et al. Review: feeding conserved forage to horses: recent advances and recommendations. Animal 2017;11:958–67.

[65] O'Kane SM, Mulhern MS, Pourshahidi LK, Strain JJ, Yeates AJ. Micronutrients, iodine status and concentrations of thyroid hormones: a systematic review. Nutr Rev 2018;76:418–31.

[66] Tripathi MK, Mishra AS. Glucosinolates in animal nutrition : a review. Anim Feed Sci Technol 2007;132:1–27.

[67] Ellis AD, Longland AC, Coenen M, Miraglia N. The impact of nutrition on the health and welfare of horses. 5th Eur. Work. Equine Nutr., Cirencester, UK: 2010.

[68] Cymbaluk N. Feeding horses. Saskatchewan Agriculture; 2015.

[69] Goer R, Coenen M, Harris PA. Equine Applied and Clinical Nutrition E-Book: Health, Welfare and Performance. 1st ed. Elsevier Health Sciences; 2013.

[70] Abbott C, Prendergast J. Histological variations in animal thyroids in western canada. Can Med Assoc J 1934;31:465–73.

[71] Schlotthauer CF. Incidence and types of disease of the thyroid gland of adult horses. JAVMA 1931;78:211–8.

[72] Delesalle C, Bruijn M, Wilmink S, Vandendriessche H, Mol G, Boshuizen B, et al. White muscle disease in foals: focus on selenium soil content. A case series. BMC Vet Res 2017;13.

[73] Van De Weyer L, Waldner CL. Geographic determinants of copper and molybdenum concentrations in serum at the end of the grazing season and associations with reproductive performance in beef cows from western canada. Can J Anim Sci 2011;91:423–31.

[74] Van De Weyer LM, Hendrick S, Waldner CL. Serum micronutrient concentrations in beef cows before and after the summer grazing season. Can J Anim Sci 2010;90:563–74.

[75] McColl JJ, Bubar CJ, Hall LM. Weeds of the prairies. Alberta. Alberta Agriculture, Food, and Rural Development; 2000.

[76] Jansson A, Dahlborn K. Effects of feeding frequency and voluntary salt intake on fluid and electrolyte regulation in athletic horses. J Appl Physiol 1999;86:1610–6.

[77] Keyes JD. Salt, Which Color Is Right? 2012.

[78] Patrick L. Iodine: deficiency and therapeutic considerations. Altern Med Rev 2008;13:116–27.

[79] Schöne F, Rajendram R. Iodine in farm animals. Compr Handb Iodine 2009:151–70.

[80] World Health Organization. Assessment of the iodine deficiency disorders and monitoring their elimination: a guide for programme managers. 3rd ed. WHO press; 2007. https://doi.org/ISBN 978 92 4 159582 7.

[81] Li Q, Mair C, Schedle K, Hammerl S, Schodl K, Windisch W. Effect of iodine source and dose on growth and iodine content in tissue and plasma thyroid hormones in fattening pigs. Eur J Nutr 2012;51:685–91.

[82] Miller JK, Swanson EW. Metabolism of ethylenediaminedihydriodide and sodium or potassium iodide by dairy cows. J Dairy Sci 1973;56:378–84.

[83] Flachowsky G, Franke K, Meyer U, Leiterer M, Schöne F. Influencing factors on iodine content of cow milk. Eur J Nutr 2014;53:351–65.

[84] Schöne F, Spörl K, Leiterer M. Iodine in the feed of cows and in the milk with a view to the consumer's iodine supply. J Trace Elem Med Biol 2017;39:202–9.

[85] Hetzel BS. Iodine deficiency and its prevention. vol. 4. Second Edi. Elsevier; 2016.

[86] Akgöl E, Tutkun E, Yilmaz H, Yilmaz FM, Gunduzoz M, Bal CD, et al. Alterations of thyroid hormone levels in cadmium exposure. J Clin Anal Med 2017;8:202–6.

[87] Sarne D. Effects of the environment, chemicals and drugs on thyroid function. In: Feingold K, Anawalt B, Boyce A, et al., editors. Endotext, South Dartmouth, MA: MDText.com, Inc.; 2016, p. 2000.

[88] Du Toit J, Casey NH. Iodine as an alleviator of bromine toxicity in thyroid, liver and kidney of broiler chickens. Livest Sci 2012;144:269–74.

[89] Jensen GKS, Rostron BJ, Duke MJM, Holmden C. Chemical profiles of formation waters from potash mine shafts, Saskatchewan. Summ Investig 2006;1:1–8.

[90] Kvíčala J, Havelka J, Němec J, Zeman V. Selenium and rubidium changes in subjects with pathologically altered thyroid. Biol Trace Elem Res 1992;32:253–8.

[91] Waugh DT. Fluoride exposure induces inhibition of sodium/iodide symporter (NIS) contributing to impaired iodine absorption and iodine deficiency: Molecular mechanisms of inhibition and implications for public health. Int J Environ Res Public Health 2019;16. https://doi.org/10.3390/ijerph16061086.

[92] Choubisa S, Mishra G, Sheikh Z, Bhardwaj B, Mali P, Jaroli V. Toxic effects of fluoride in domestic animals. Adv Pharmacol Toxicol 2011;12:29–37.

[93] Arthur J, Beckett G. Thyroid function. Br Med Bull 1999;55:658–68.

[94] Hess SY. The impact of common micronutrient deficiencies on iodine and thyroid metabolism: the evidence from human studies. Best Pract Res Clin Endocrinol Metab 2010;24:117–32.

[95] Vásquez-Garibay. Iodine deficiency in relation to iron deficiency and parasitosis: effect of iron status and parasites on iodine deficiency disorders. In: Priddy V, Burrows G, Watson R, Al. E, editors. Compr. Handb. Iodine. Nutr. Biochem. Pathol. Ther. Asp. 1st ed., Elsevier; 2009, p. 499–511.

[96] Cymbaluk N, Christensen D. Copper, zinc and manganese concentrations in equine liver, kidney and plasma. Can Vet J = La Rev Vet Can 1986;27:206–10.

[97] Cymbaluk N, Schryver H, Hinz H. Influence of dietary molybdenum on coppermetabolism in ponies. J Nutr 1981;111:96–106.

[98] Walter Swerczek T. Effects of nitrate and pathogenic nanoparticles on reproductive losses, congenital hypothyroidism and musculoskeletal abnormalities in mares and other livestock: new hypotheses. Anim Vet Sci 2019;7:1.

[99] Eisenbrand G, Gelbke HP. Assessing the potential impact on the thyroid axis of environmentally relevant food constituents/contaminants in humans. Arch Toxicol 2016;90:1841–57.

[100] Sanchez CA, Blount BC, Valentin-Blasini L, Lesch SM, Krieger RI. Perchlorate in the feed-dairy continuum of the southwestern united states. J Agric Food Chem 2008;56:5443–50.

[101] Breuhaus B, Refsal KR, Beyerlein SL. Measurement of free thyroxine concentrations in horses by equilibrium dialysis. J Vet Intern Med 2006;20:371–6.

[102] Kemppainen RJ, Behrend EN. Diagnosis of canine hypothyroidism. Perspectives from a testing laboratory. Vet Clin North Am Small Anim Pract 2001;31:951–62.

[103] Meredith TB, Dobrinski I. Thyroid function and pregnancy status in broodmares. J Am Vet Med Assoc 2004;224:892–4.

[104] Alexander EK, Heering JP, Benson CB, Frates MC, Doubilet PM, Cibas ES, et al. Assessment of nondiagnostic ultrasound-guided fine needle aspirations of thyroid nodules.

J Clin Endocrinol Metab 2002;87:4924–7.

[105] Park VY, Lee HS, Kim EK, Kwak JY, Yoon JH, Moon HJ. Frequencies and malignancy rates of 6-tiered bethesda categories of thyroid nodules according to ultrasound assessment and nodule size. Head Neck 2018;40:1947–54.

[106] Perillo A, Passantino G, Passantino L, Cianciotta A, Mastrosimini AM, Iacovazzi P, et al. First observation of an hashimoto thyroiditis-like disease in horses from eastern europe: histopathological and immunological findings. Immunopharmacol Immunotoxicol 2005;27:241–53.

[107] Dória RGS, Canola PA, Ribeiro G, Di Filipo PA, Dias DPM, Valadão CAA. Equine iatrogenic hypothyroidism due to iodine excess: case report. Arq Bras Med Vet e Zootec 2008;60:521–4.

[108] Osame S, Ichi S. Clinicopathological observations in thoroughbred foals with enlarged thryoid gland. Japanese Vet Med Sci 1994;56:771–2.

[109] Rodenwold B, Simms B. . Iodine for brood mares. J Anim Sci 1935;1935:89–92.

[110] Diel de Amorim M, Iv NTM, Card C. Primary hypothyroidism in mares from a herd affected with congenital hypothyroidism. Proc 61st Annu Conv Am Assoc Equine Pr., Las Vegas, Nevada: 2015, p. 17.

[111] Breuhaus BA. Thyroid-stimulating hormone in adult euthyroid and hypothyroid horses. J Vet Intern Med 2002:109–15.

[112] Manna D, Roy G, Mugesh G. Antithyroid drugs and their analogues: synthesis, structure, and mechanism of action. Acc Chem Res 2013;46:2706–15.

[113] Johnson P, Messer N, Ganjam V, Thompson D, Refsal K, Loch W, et al. Effects of propylthiouracil and bromocryptine on serum concentrations of thyrotrophin and thyroid hormones in normal female horses. Equine Vet J 2003;35:296–301.

[114] Frank N, Sojka J, Messer NT. Equine thyroid dysfunction. Vet Clin North Am - Equine Pract 2002;18:305–19.

[115] Himler M, Hurcombe SDA, Griffin A, Barsnick RJ, Rathgeber RA, MacGillivray KC, et al. Presumptive nonthyroidal illness syndrome in critically ill foals. Equine Vet J 2012;44:43–7. https://doi.org/10.1111/j.2042-3306.2011.00480.x.

[116] Nelson RW. Diagnosis and treatment of canine hypothyroidism. Vet Q 1996;18:29–31.

[117] Troillet A, Bottcher D, Brehm W, Scharner D. Retrospective evaluation of

hemithyroidectomy in 14 horses. Vet Surg 2016;45:949–54.

[118] Koikkalainen K, Knuuttila A, Karikoski N, Syrjä P, Hewetson M. Congenital hypothyroidism and dysmaturity syndrome in foals: first reported cases in europe. Equine Vet Educ 2014;26:181–9.

[119] Sommardahl CS, Frank N, Elliott SB, Webb LL, Refsal KR, Denhart JW, et al. Effects of oral administration of levothyroxine sodium on serum concentrations of thyroid gland hormones and responses to injections of thyrotropin-releasing hormone in healthy adult mares. Am J Vet Res 2005;66:1025–31.

[120] Frank N, Buchanan BR, Elliott SB. Effect of long-term oral administration of levothyroxine sodium on serum thyroid hormon concentrations, clinicopathologic variables, and echocardiographic measurments in healthy adult horses. Am J Vet Res 2008;69:68–75.

[121] Lester GD. Maturity of the neonatal foal. Vet Clin Equine 2005;21:333–55.

[122] Panzani S, Comin A, Galeati G, Romano G, Villani M, Faustini M, et al. How type of parturition and health status influence hormonal and metabolic profiles in newborn foals. Theriogenology 2012;77:1167–77. https://doi.org/10.1016/j.theriogenology.2011.10.023.

[123] Pirrone A, Panzani S, Govoni N, Castagnetti C, Veronesi MC. Thyroid hormone concentrations in foals affected by perinatal asphyxia syndrome. Theriogenology 2013;80:624–9. https://doi.org/10.1016/j.theriogenology.2013.06.003.

[124] Zimmermann MB, Boelaert K. Iodine deficiency and thyroid disorders. Lancet Diabetes Endocrinol 2015;3:286–95.

[125] Leung AM, Braverman LE. Iodine-induced thyroid dysfunction. Curr Opin Endocrinol Diabetes Obes 2012;19:414–9.

[126] Allen A, Doige CE, Fretz PB, Townsend HGG. Hyperplasia of the thyroid gland and concurrent musculoskeletal deformities in western canadian foals : reexamination of a previously described syndrome. Can Vet J 1994;35:31–8.

[127] Vidal S, Horvath E, Kovacs K, Cohen SM, Lloyd R V., Scheithauer BW. Transdifferentiation of somatotrophs to thyrotrophs in the pituitary of patients with protracted primary hypothyroidism. Virchows Arch 2000;436:43–51.

[128] Allen A. Congenital hypothyroidism in horses: looking back and looking ahead. Equine Vet Educ 2014;26:190–3.

[129] Bischoff KL. Glucosinolates. Nutraceuticals Effic. Saf. Toxic., Elsevier Inc.; 2016, p. 551–4.

[130] Ishida M, Hara M, Fukino N, Kakizaki T, Morimitsu Y. Glucosinolate metabolism, functionality and breeding for the improvement of brassicaceae vegetables. Breed Sci 2014;64:48–59.

[131] Merillon J-M, Ramawat KG. Glucosinolates. 1st ed. Switzerland: Springer; 2017.

[132] International Agency for Research of Cancer. Glucosinolates, isothiocyanates and indoles. IARC Handb Cancer Prev 2004:254.

[133] Chandra A, Debnath A, Tripathy S, Goswami H, Mondal C, Chakraborty A, et al. Environmental factors other than iodine deficiency in the pathogenesis of endemic goiter in the basin of river Ganga and Bay of Bengal, India. BLDE Univ J Heal Sci 2016;1:33–8.

[134] Laarveld B, Brockman R, Christensen D. The effects of the level of iodine in canola meal concentrate on milk iodine and thiocyanate content and thyroid function in dairy cows. Can J Anim Sci 1981;61:625–32.

[135] Laarveld B, Brockman R, Christensen D. The effects of tower and midas rapeseed meals on milk production and concentrations of goitrogens and iodide in milk. Can J Anim Sci 1981;61:131–9.

[136] Chandra AK, Singh LH, Ghosh S, Pearce EN. Role of bamboo-shoot in the pathogenesis of endemic goiter in manipur, north east india. Endocr Pract 2013;19:36–45.

[137] Franke K, Meyer U, Wagner H, Hoppen HO, Flachowsky G. Effect of various iodine supplementations, rapeseed meal application and two different iodine species on the iodine status and iodine excretion of dairy cows. Livest Sci 2009;125:223–31.

[138] Kursa J, Travnicek J, Rambeck W, Kroupova V, Vitovec J. Goitrogenic effects of extracted rapeseed meal and nitrates in sheep and their progeny. Vet Med Czech 2000;45:129–40.

[139] Bobek S, Ewy Z, Kahl S, Pyska H. Problems of iodine and thiocyanate in domestic animals in goitrogenic areas of southern Poland. Endokrynol Pol 1992;43:70–82.

[140] Papas A, Ingalls J, Campbell L. Studies on the effects of rapeseed meal on thyroid status of cattle, glucosinolate and iodine content of milk and other parameters. J Nutr 1979;106:1129–39.

[141] Oliveira K de, Furtado CE, Graça EP da. Performance and blood parameters of growing

equine fed diets with different levels of canola meal. Rev Bras Zootec 2001;30:174–80.
[142] Cymbaluk N. Using canola meal in growing draft horse diet. Equine Pract 1991;12:13–9.
[143] Schöne F, Leiterer M, Hartung H, Jahreis G, Tischendorf F. Rapeseed glucosinolates and iodine in sows affect the milk iodine concentration and the iodine status of piglets. Br J Nutr 2001;85:659–70.
[144] Śmiechowska A, Bartoszek A, Namieśnik J. Determination of glucosinolates and their decomposition products-indoles and isothiocyanates in cruciferous vegetables. Crit Rev Anal Chem 2010;40:202–16.
[145] Song L, Morrison JJ, Botting NP, Thornalley PJ. Analysis of glucosinolates, isothiocyanates, and amine degradation products in vegetable extracts and blood plasma by LC-MS/MS. Anal Biochem 2005;347:234–43.
[146] Bruce G, Corey L, Mandel J, Pleus R. Urinary nitrate, thiocyanate, and perchlorate and serum thyroid endpoints based on NHANES 2001 to 2002. J Occup Environ Med 2013;55:52–8.
[147] Gawrylash SK. Thyroid hyperplasia and musculoskeletal deformity in a standardbred filly in ontario. Can Vet J 2004;45:424–6.
[148] USDA Natural Resources Conservation Service. Introduced, invasive, and noxious plants n.d. https://plants.sc.egov.usda.gov/java/invasiveOne?queryParam=comname&sort=comname&startChar=M.
[149] IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans: allyl compounds, aldehydes, epoxides and peroxides. vol. 36. Lyon, International Agency for Research on Cancer; 1985.
[150] Judson HG, Barrell GK. The incidence of goitre in newborn lambs from ewes fed fodder radish , rape or Italian ryegrass with or without iodine supplementation. Proc. New Zeal. Soc. Anim. Prod., vol. 71, 2011, p. 66–70.
[151] Sutton E, Stredwick R. Acceptance of rapeseed meal (cv. candle) by horses. Can J Anim Sci 1979;59:819–20.
[152] Fazio E, Medica P, Cravana C, Ferlazzo A. Total and free iodothyronines profile in the donkey (equus asinus) over a 12-month period. Acta Vet Brno 2012;81:239–44.
[153] Sticker LS, Thompson DL, Gentry LR. Pituitary hormone and insulin responses to

infusion of amino acids and n-methyl-d,l-aspartate in horses. J Anim Sci 2001;79:735–44.

[154] Oliveira RDL, Dhingra OD, Lima AO, Jham GN, Berhow MA, Holloway RK, et al. Glucosinolate content and nematicidal activity of brazilian wild mustard tissues against meloidogyne incognita in tomato. Plant Soil 2011;341:155–64.

[155] Qu CM, Li SM, Duan XJ, Fan JH, Jia LD, Zhao HY, et al. Identification of candidate genes for seed glucosinolate content using association mapping in brassica napus l. Genes (Basel) 2015;6:1215–29.

[156] Thompson DL, Nett TM. Thyroid stimulating hormone and prolactin secretion after thyrotropin releasing hormone administration to mares: dose response during anestrus in winter and during estrus in summer. Domest Anim Endocrinol 1984;1:263–8.

[157] Paul T, Witorsch RJ, Pino S, Chipkin S, Ingbar SH, Braverman LE. The effect of small increases in dietary iodine on thyroid function in euthyroid subjects. Metabolism 1988;37:121–4.

[158] Grace ND, Pearce SG, Firth EC, Fennessy PF. Concentrations of macro- and micro-elements in the milk of pasture-fed thoroughbred mares. Aust Vet J 1999;77:177–80.

[159] Doureau M, Martin-Roset W. Animals that produce dairy foods – horse. Encycl. Dairy Sci. 2nd ed, 2011, p. 358–64.

[160] Geor R, Harris P, Coenen M. Macro and trace elements in equine nutrition. In: RJ G, M C, PA H, editors. Equine Appl. Clin. Nutr. Heath, Welf. Perform., Toronto: Saunders; 2013, p. 220–4.

[161] National Research Council NRC. Nutrient requirements of horses. 6th revise. Washington, DC, USA: The National Academies Press; 2007.

[162] Schryver HF, Parker MT, Daniluk PD, Pagan KI, Williams J, Soderholm L V, et al. Salt consumption and the effect of salt on mineral metabolism in horses. Cornell Vet 1987;77:122—131.

[163] Kavazis AN, Kivipelto J, Ott EA. Supplementation of broodmares with copper, zinc, iron, manganese, cobalt, iodine, and selenium. J Equine Vet Sci 2002;22:460–4.

[164] Csapb-kiss Z, Stefler J, Martin TG, Makray S, Csap J. Composition of mares' colostrum and milk . protein content, amino acid composition and contents of macro- and micro-elements. Int Dairy J 1995;5:403–15.

[165] Coleman MC, Whitfield-Cargile C. Orthopedic conditions of the premature and dysmature

foal. Vet Clin Equine 2017;33:289–97.

[166] Doige CE, McLaughlin BG. Hyperplastic goitre in newborn foals in western canada. Can Vet J 1981;22:42–5.

[167] Drew B, Barber WP, Williams DG. The effect of excess dietary iodine on pregnant mares and foals. Vet Rec 1975;97:93—95.

[168] Eroksuz H, Eroksuz Y, Ozer H, Ceribasi AO, Yaman I, Ilhan N. Equine goiter associated with excess dietary iodine. Vet Hum Toxicol 2004;46:147—149.

[169] Duncan AJ, Milne JA. Effects of oral administration of brassica secondary metabolites, allyl cyanide, allyl isothiocyanate and dimethyl disulphide, on the voluntary food intake and metabolism of sheep. Br J Nutr 1993;70:631–45.

[170] Pedraza PE, Obregon MJ, Escobar-Morreale HF, Del Rey FE, De Escobar GM. Mechanisms of adaptation to iodine deficiency in rats: thyroid status is tissue specific. Its relevance for man. Endocrinol 2006;147:2098–108.

[171] Pattanaik AK, Khan SA, Varshney VP, Bedi SPS. Effect of iodine level in mustard (brassica juncea) cake-based concentrate supplement on nutrient utilisation and serum thyroid hormones of goats. Small Rumin Res 2001;41:51–9.

[172] Irvine G. Thyroxine secretion rate in the horse in various physiological states. vol. 39. 1967.

[173] Alexander S, Irvine C, Evans M. Inter-relationships between the secretory dynamics of thyrotrophin-releasing hormone, thyrotrophin and prolactin in periovulatory mares: effect of hypothyroidism. J Neuroendocrinol 2004;16:906–15.

[174] Breuhaus B. Thyroid hormone and thyrotropin concentrations and responses to thyrotropin-stimulating hormone in horses with PPID compared with age-matched normal horses. J Equine Vet Sci 2019;75:35–40.

[175] Eastman CJ. Screening for thyroid disease and iodine deficiency. Pathology 2012;44:153–9.

[176] Hines M, Gay C, Talcott C. Congenital hypothyroidism and dysmaturity syndrome of foals: diagnosis and possible risk factors. 15th Am. Coll. Vet. Intern. Med., Lake Buena Vista, Lakewood, Colorado.: ACVIM; 1997, p. 363–4.

[177] National Research Council. 6th ed., Washington, D.C.: The National Academies; n.d.

[178] Katagiri R, Asakura K, Uechi K, Masayasu S, Sasaki S. Iodine excretion in 24-hour urine

collection and Its dietary determinants in healthy japanese adults. J Epidemiol 2016;26:613–21.

[179] Chavatte-Palmer P, Peugnet P, Robles M. Developmental programming in equine species: relevance for the horse industry. Anim Front 2017;7:48–54.

[180] Lanham SA, Fowden AL, Roberts C, Cooper C, Oreffo ROC, Forhead AJ. Effects of hypothyroidism on the structure and mechanical properties of bone in the ovine fetus. J Endocrinol 2011;210:189–98.

[181] Coleman MC. Orthopedic conditions of the premature and dysmature foal incomplete ossification premature dysmature hypothyroid cuboidal bones 2017;33:289–97.

[182] Choi EJ, Zhang P, Kwon H. Determination of goitrogenic metabolites in the serum of male wistar rat fed structurally different glucosinolates. Toxicol Res 2014;30:109–16.

[183] Langer P, Stolc V. Notes and comments goitrogenic activity of allylisothiocyanate-a widespread natural mustard oil. n.d.

[184] Kostogrys R, Pisulewski P, Pecio A, Filipiak-Florkiewicz A. Goitrogenic effects of allylisothiocyanate, nitrate, and nitrite in rats and alleviating propperties of iodine and selenium supplements. Polish J Food Nutr Sci 2010;60:165–73.

[185] Mazumder A, Dwivedi A, Plessis J Du. Sinigrin and its therapeutic benefits. Molecules 2016;21.