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Original Study **OS1(1-9)**

Effect of increasing exercise levels, feeding-housing regimes and sex on the total and free iodothyronines of trained horses

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Abstract

The aim of the present study was to assess and compare the effect of increasing exercise levels of thirty-six trained Dutch Warmblood horses fed a isoenergetic high versus low fiber content on the circulating Thyroid hormones (THs): triiodothyronine (T_3), thyroxine (T_4), free triiodothyronine (fT_3) and free thyroxine (fT_4) concentrations. The training schedule was carried out in three consecutive phases, according to three exercise levels (light, medium and medium-heavy) performed on trainingsmill. Each phase lasted 4 weeks. At week 4 of each phase a standardized exercise test (SET) on treadmill was performed. Blood samples were collected at rest (baseline values), and after 15, 30 and 60 min after SET, according to different phases. Horses were randomly distinguished in two groups of 18 subjects. One group of horses was fed a high fiber diet (HF group) and one group was fed a low fiber diet (LF group).

Results showed that in basal conditions diet influenced significantly fT_3 values, with the highest concentration in HF, and fT_4 values with the highest concentrations in LF group. The time influenced significantly THs in both groups, with the highest values at June for T_3, and at October for T_4, fT_3 and fT_4. A significant correlation between T_4 and fT_4 was observed in basal conditions.

In exercise conditions, diet influenced only fT_4 values, with thehighest concentrations in LF group. The time influenced significantlyT_4 andfT_4 concentrations, with the highest values at 15 min for fT_4 and at 30 min for T_4 values and the lowest values at 60 min for both hormones. Significant correlations between T_3 and fT_3, as well as T_4 and fT_4 were observed in exercise conditions.

The evaluation of THs output, according to diet contents, could mirror a quantitative and qualitative interplay between thyroid function and increasing exercise level of trained horses.

KeyWords: Exercise, time, diet, sex, thyroid hormones

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Introduction

Thyroid hormones are known to be involved in key metabolic pathways in the regulation of basal, lipid and glucose metabolisms and energy expenditure, growth and maturation of organ systems (1,2).Different physiologic states can alter thyroid hormone synthesis, metabolism or binding, resulting in changed circulating thyroid hormone concentrations; in the horse, these include strenuous exercise and fasting. Diets high in energy, protein, zinc and copper have also resulted in alterations in circulating concentrations of thyroid hormones in horses (2). The complex interplay between the effects of diet contents on welfare and performance of sport horses has become of great interest in the scientific community (3,4). Moreover, hormonal, metabolic and behavioral patterns in horses, in response to different feeding-housing regimes, were also studied (5-7) and the use of sport horses as “competition tools”, according to the potential effects of stress on their performance was defined (8-10). Although different researches were developed to assess the thyroid responses of sport horses during different equestrian discipline, including competitive and not competitive events (9,10), there are few and fragmentary literature data on impact of a combination of exercise and diet on horse’s thyroid function (11,12).

The evaluation of thyroid response, measuring the free fractions of thyroid hormones, alone or in conjunction with measurement of total amounts of hormone, provides more useful information than does measurement of total amounts of thyroid hormones alone (2). The hypothesis of the current study was that diet contents could play an important role on the thyroid homeostasis of sport horses during training schedule, according to the horses’performance.

Here, we report the results of a study designed to characterize the mid-term thyroid response to a supervised exercise plus diet program of Warmblood horses, adding a new segment of scientific literature in this mean topic.

Material and methods

Horses

As part of a larger study into exercise physiology and welfare of the Dutch Warmblood sport horses, the current study comprised thirty-sixhealthy horses (18 mares and 18 geldings), aged 3 years, weighting 554 ± 42 kg, housed in individual stables of 3 x 3.5 m, with two stable blocks on a hemp (phases 1 and 2) and wood shavings bedding (phase 3). All horses were reared under similar conditions and were accustomed to individual housing, trainingsmill and treadmill work during a previous period of 4 months, reaching a light fitness level (20 minutes walk, 8 minutes trot on the trainingsmill). The horses were trained under the supervision of professional trainers.

The training schedule was carried out in three consecutive phases, according to three exercise levels (light, medium and medium-heavy) and was performed on trainingsmill. Each phase lasted 4 weeks. In week 4 of each phase a standardized exercise test (SET) was performed.

Phase 1 (light) consisted of: 18 min/day of walk, 10 min/day of trot, 5 min/day of canter, for a total of 38 min/day. Phase 2 (medium) consisted of: 22 min/day of walk, 15 min/day of trot, 8 min/day of canter, for a total of 45 min/day. Phase 3 (medium-heavy) consisted of: 21 min/day of walk, 36 min/day of trot, 15 min/day of canter, for a total of 72 min/day.

At the end of each phase horses were submitted to SET on the treadmill, for a total of time spent paired to 15 min. Speed in m/s ranged between ±1.8 to ±4.0. Heart rate and blood lactate concentrations showed aerobic capacity of the horses during phases 1-2, with heart rates varying between 150 and 160 beats/min, and blood lactate concentrations not exceeding 2 mM/L (13). On the contrary, phase 3 resulted in a greater dependence on the muscle anaerobic energy system.

Diet

To determine the effect of dietary fiber contents, horses were fed two isoenergetic diets for 4 weeks. Horses were randomly distinguished in two groups of 18, with each group containing 9 mares and 9 geldings. One group of horses was fed on a high fiber diet (HF group) (Dry matter ratio - concentrate: silage =1:4), and one group was fed on a low fiber diet (LF group) (Dry matter ratio - concentrate: silage =4:1). At the end of phase 3, all horses were kept in two groups according to sex (each consisting of 50% former HF group and 50% former LF group) on grassland for 3 months. The feeding regime for the “control” diet was, therefore, grass and silage.

All procedures involved in the experimental protocol were approved by Ethical Animal Commission, ID-Lelystad, the Netherlands.

Sample collection

Blood samples were collected from jugular vein at rest, (rest sample: T0), at 11.00 a.m., immediately after 15 min SET (T1 sample) in phase 1, and 15 min later (recovery sample, after 30 minT2 sample) in phase 2. In phase 3 only, blood samples were taken exactly 60 min after training on the trainingsmill (at the end of final bout trotting: T3 sample). Horses were accustomed to clinical routines and blood collections. All samples were collected into evacuated tubes (Venoject, Terumo® Belgium) and were immediately refrigerated at 4°C after collection; the samples were subsequently (within 1 h) centrifuged for 15 min at 1,500 g. Serum was harvested and stored in polystyrene tubes at -20°C until assayed for serum total and free iodothyronine concentrations.

Hormonal analyses

Serum total (T_3, T_4)and free iodothyronine (fT_3, fT_4) concentrations were analyzed in duplicate using commercial immunoenzymatic assays (EIA, RADIM, Rome, Italy). The method is based on a competitive EIA, and the reagents were prepared as described in the manufacturer’s protocol. The assay’s sensitivity was as follows: 0.6 ng/ml for T_3, 4.5 ng/ml for T_4, 0.16 pg/ml for fT_3 and < 1 pg/ml for fT_4.

The respective intra- and interassay coefficients of variation (CVs) were as follows: 7.3% and 11.4% for T_3, 2.3%, and 5.7% for T_4, 4.2% and 11.9 % for fT_3, and 6.6% and 9.6 % for fT_4.

Statistical analyses

To account for the study design, a mixed model analysis of variance (XLSTAT Addinsoft v. 2014.4.03) with the fixed effects of Diet (HF vs. LF), Sex (Geldling and Female) and Time (Months: June and October; Exercise time (immediately pre: T0, immediately post: T1, 15 min post exercise: T2, and 24h post exercise: T3) was applied. In the model, three fixed factors (Diet, Time, Sex) and the interaction factor Diet*Time*Sex were considered.

Least Squares Means (LSM) and standard error of the mean (SEM) were calculated. Comparisons between LSM were performed using the Tukey test. Differences were considered significant for *P*<0.05.To examine the correlations between hormones, Spearman's correlation coefficient was used.

Result

Hormonal response in basal condition (Months: from June to October)

As regards the trend of THsin relation to the Diet (Table 1), results showed that the diet did not influence T_3 and T_4 concentrations. The fT_3 values were significantly (P=0.006) influenced, with higher (P<0.05) mean concentrations in HF group than those observed in LF group. The fT_4 values were significantly (P=0.014) influenced, showing significant (P<0.05) higher mean concentrations in LF group than those observed in HF group.

Table 1 - Effect of diet on thyroid hormone concentrations in basal condition (June and October).†

Groups‡	**T_3 (nmol/L)**		**T_4 (nmol/L)**		**fT_3 (pmol/L)**		**fT_4 (pmol/L)**	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
HF	2.76	0.15	58.48	3.93	4.68 ^{a}	0.33	25.48 ^{b}	1.02
LF	2.62	0.15	67.90	4.28	3.62 ^{b}	0.24	28.95 ^{a}	0.90

T_3= Triiodothyronine Hormone; T_4= Thyroxine Hormone; fT_3= free Triiodothyronine Hormone; fT_4 = free Thyroxine Hormone

† Values are given as least square mean (LSM) ± standard error of the mean (SEM).

‡ Groups= HF = high-fibre content diet; LF = low-fibre content diet.

a, b Means within a column with different superscripts indicate a significant difference at *P*< 0.05 using the Tukey test.

Related to the Time (months), T_3 (P=0.002), T_4 (P=0.001), fT_3(P=0.030) and fT_4 (P=0.007) concentrations were significantly influenced by the time (June and October) (Table 2), showing in both groups the highest values at June for T_3, and at October for T_4, fT_3 and fT_4.

Table 2 - Effect of time on thyroid hormone concentrations in basal condition (June and October).†

Time ‡	**T_3 (nmol/L)**		**T_4 (nmol/L)**		**fT_3 (pmol/L)**		**fT_4 (pmol/L)**	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
June	3.07 ^{a}	0.15	47.27 ^{b}	1.98	3.59 ^{b}	0.15	25.35 ^{b}	0.77
October	2.33 ^{b}	0.15	79.11 ^{a}	4.07	4.56 ^{a}	0.35	29.08 ^{a}	1.02

T_3= Triiodothyronine; T_4= Thyroxine; fT_3= free Triiodothyronine; fT_4 = free Thyroxine

† Values are given as least square mean (LSM) ± standard error of the mean (SEM).

‡ Time = Blood sampling at T0 before the exercise in basal condition.

a, b Means within a column with different superscripts indicate a significant difference at *P*< 0.05 using the Tukey test.

On the whole trial period, sex did not influence T_3 (P=0.542), nor T_4 (P=0.227), neither fT_3 (P=0.466) and fT_4 (P= 0.792) concentrations.

The interaction Diet*Time*Sex showed no significant differences for all variables and hormones.

A significant correlation between T_4 and fT_4 (r= 0.54; p<0.001) was observed.

Hormonal response in exercise condition (sampling time: from T0 to T3)

As regards the trend THs in relation to the Diet, in exercise condition (Table 3), it did not influence T_3 nor T_4 and fT_4 concentrations. The fT_4 values were significantly (P<0.001) influenced, with higher (P<0.05) mean concentrations in LF group than those observed in HF group.

Table 3 - Effect of diet on thyroid hormone concentrations in exercise condition.[†]

Groups[‡]	**T_3 (nmol/L)**		**T_4 (nmol/L)**		**fT_3 (pmol/L)**		**fT_4 (pmol/L)**	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
HF	3.48	0.09	49.94	2.05	3.84	0.12	16.60[b]	1.15
LF	3.27	0.06	54.36	2.08	3.90	0.13	25.35[a]	1.15

T_3= Triiodothyronine; T_4= Thyroxine; fT_3= free Triiodothyronine; fT_4 = free Thyroxine
[†] Values are given as least square mean (LSM) ± standard error of the mean (SEM).
[‡] Groups= HF = high-fibre content diet; LF = low-fibre content diet.
[a, b]Means within a column with different superscripts indicate a significant difference at *P*< 0.05 using the Tukey test

Related to the Time (T0 = at rest, pre- exercise, T1 = after 15 min post exercise, T2 = after 30 min post exercise, T3 = after 60 min post exercise), the time did not influence T_3 and fT_3 concentrations. The T_4 (P=0.001) and fT_4 (P=0.001) concentrations were significantly influenced by the time (Table 4), showing the lowest values at T3 for both hormones, and the highest values at T2 for T_4 values and at T1 for fT_4 values.

Table 4 - Effect of time on thyroid hormone concentrations before, during and after exercise.[†]

Time[‡]	**T_3 (nmol/L)**		**T_4 (nmol/L)**		**fT_3 (pmol/L)**		**fT_4 (pmol/L)**	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
T0	3.04	0.15	47.27	1.98	3.73	0.15	25.35	0.77
T1	3.62	0.19	58.09	2.70	3.96	0.18	29.72[a]	1.15
T2	4.17	0.21	64.42[a]	3.19	5.51	0.26	24.45	1.67
T3	2.70	0.12	38.84[b]	1.78	3.76	0.13	9.75[b]	0.64

T_3= Triiodothyronine; T_4= Thyroxine; fT_3= free Triiodothyronine; fT_4 = free Thyroxine
[†] Values are given as least square mean (LSM) ± standard error of the mean (SEM).
[‡] Time = Blood sampling at T0 before the exercise, at T1 immediately after exercise, at T2 after 15 min, at T3 after 24 h.
[a, b]Means within a column with different superscripts indicate a significant difference at *P*< 0.05 using the Tukey test.

On the whole trial period, Sex did not influence T_3 (P=0.491), nor T_4 (P=0.745), neither fT_3 (P=0.216) and fT_4 (P= 0.375) concentrations.

The interaction Diet*Time*Sex showed no significant differences for all variables and hormones.

Significant correlations between T_3 and fT_3 (r= 0.16; p=0.04), as well as T_4 and fT_4 (r= 0.41; p<0.001) were observed.

Discussion

Exercise can influence thyroid hormones, and the measurement and interpretation of total and free iodothyronine concentrations during standardized incremental exercise regimen could be a key of their interplay. The comparison of obtained data with published ranges for horsesrevealed that thyroid hormone concentrations were in agreement with the physiological reference ranges reported in literature for normal and sport horses (9, 10, 14).

Basal conditions: The significant effect of diet in basal conditions was observed only on the free iodothyronines, with the highest values of fT_3 in HF group, and of fT_4 in LF group. This result did not confirm data reported in Thoroughbred geldings, in which the meal feeding produced a significant increase in T_4 and T_3 concentrations when horses were adapted to the nutritionally adequate high-roughage ration (70% roughage, 30% concentrate), and/or a nutritionally adequate high-concentrate ration (40% roughage, 60% concentrate) diets (11). In the current study, an adequate and isoenergetic nutritionally content of concentrate was used: silage was paired to 1:4 (HF group), and content of concentrate: silage was paired to 4:1 (LF group), confirming that responses to meal feeding in horses may also be affected by the dietary roughage toconcentrate ratio (12). On the other hand, other authors reported that thyroid hormones in mares,fed diets of grass hay and a corn/cottonseed hull-based supplement formulated to contain either 100% (control) or 50% (restricted) of the protein and (or) energy requirements, were not influenced by restriction of protein and/or energy (15). The existence of contradictory results thus indicates that further studies are needed to elucidate the emerging role of diet according to the energetic expenditure, in relation to the resting and exercise conditions.

The significant effect of diet on the fT_4 values in both basal and exercise conditions, with the constant highest values always in LF group, could indicate its wide and active metabolic role, confirming that this free fraction represents one of the biologically active hormones for tissues; thus, the ratio of fT_4 represents a primary factor for determining the fractional turnover of thyroid hormones.

The experimental design was designed as a nutritional study *per se*, applied to increasing exercise-training levels, evaluating the comparison of two commonly used feeding-housing regimens and their effects on thyroid function, in response to the different exercise phases. Given that an effect of diet was observed in both basal and exercise conditions, it is appropriate to discuss the differences in HF and LF regimes, according to the single exercise's phases.

Feeding a high concentrate low fibre diet over a period can have welfare implication for the horse; however, for many horses performing below maximum exertion levels, a diet lacking in structural fibre may be unnecessary (4). Generally, the argument that sport horses cannot perform well on a high fibre diet has pervaded. Increasing performance work in the horse is often associated with a reduction in the forage ration and an increase in concentrates, but insufficient research exists to verify this (16). As a matter of fact, a significant effect of diet was observed for fT_4, with higher values in LF than HF groups, but without different performance between the two groups.

Exercise conditions: The significant highest fT_4 and T_4 concentrations, respectively immediately 15 min after exercise and after 30 min, and their lowest concentrations after 60 min show that the thyroid response helps to give an indication of how horses are physiologically responding to training sessions, independent of the different diet composition. These results do not confirm data reported in Thoroughbred geldings submitted to moderately intense work, in which daily serum T_4 and T_3 concentrations were not affected by day, feeding level, or diet composition (12). However, serum T_3 concentrations significantly increased in response to exercise within horses fed a calorie-restricted diet designed to have 70% of the calories from the roughage source, and horses fed a calorie-restricted diet designed to have 70% of the calories from the concentrate source. The differences in thyroid hormone responses to exercise suggest that calorie source may be important in the hormonal regulation and energy metabolism of horses consuming calorie deficient diets (11). Hence, to exclude this effect, in the present study all horses were fed an isoenergetic diet in relation to exercise expenditure per phase. Energy requirements for each horse were calculated according to the Dutch Energy Evaluation System for horses, and slightadjustment for body condition was made if the score was more than ±20% of ideal body score (15). All horses were fed a minimum of 1.4 times digestible crude protein requirements. In addition, the HF group was fed an additional mineral supplement in order to adjust mineral intakes as close as possible to those levels fed in the LF group. A unified approach would help to minimize the confusion currently present and to maximize progress in this area, regarding the energy sources available to the horse, from its diet and from its body stores, at rest and while exercising.

The most important finding of the present study was that the increasing exercise level represented a major determinant of thyroid output, synchronizing the fT_4 and T_4 pathway, with respectively early and late increases after exercise and, at the same time, the long-term responses of both hormones, as already concluded (9,10).

Another point is that this response may not be necessarily related to different fibre contents and, as matter of facts, both LF and HF groups do not display any significant differences along exercise conditions. That is, for healthy trained horses, average thyroid hormone concentrations differed only between the increasing exercise levels (phases 1-3) compared to baseline values.

In a multidisciplinary approach, the modality of TH changes, according to different diets, was clearly displayed as being part of any specific “increasing exercise levels” outcome rather than a generalized feeding-metabolic response, as reported in previous studies related to a different effect of horse-feeding-regimes interaction on the hormonal and metabolic homeostasis (5). The magnitude of thyroid changes, following a period of 4 weeks of experimental feeding-regimes (LF and HF groups), suggests a relative contribution made by this different diet regime only on the fT_4 endpoints.

What is more, the significant and positive correlations between T_4:fT_4 in basal conditions, and between T_3:fT_3 and T_4:fT_4 in exercise conditions, independent of diet, time and sex, are in line with previous data , in which changes in circulating fT_3 and fT_4 concentrations generally followed those for T_3 and T_4 (9,10).

The acrophase of T_3 in June was in accordance with that observed in both barren and pregnant donkeys (26).Sex did not induced a significant effect on the THs, confirming recent data of Breuhaus (2018).

Endocrine changes may, therefore, be either the physiological responses to increasing exercise level or to feeding regime (diet) in the time points (time).

The present data do not completely fulfil the growing scientific interest on the total and free iodothyronine patterns and their interaction with diet in response to increasing exercise, but add a new segment of scientific literature in this topic on trained Dutch Warmblood horses.

Conflicts of interest: The authors declare no conflict of interest.

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