Total and free iodothyronine changes in response to transport of Equidae (Equus asinus and Equus caballus)

Esterina Fazio*, Pietro Medica, Cristina Cravana and Adriana Ferlazzo

Department of Veterinary Sciences, Polo Universitario Annunziata, 98168 Messina, Italy

* *Corresponding author at: Department of Veterinary Sciences, Polo Universitario Annunziata, 98168 Messina, Italy. Tel.: +39 90 3503 583, fax: +39 90 3503 975, e-mail: fazio@unime.it.*

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Donkey,
Free iodothyronines,
Horse,
Total iodothyronines,
Transport.

Summary
In this study the effects of short distance road transport on total and free iodothyronine changes in 12 stallions (*Equus asinus* and *Equus caballus*) were evaluated. Donkeys (n = 6) and horses (n = 6) were transported for a distance of 50 km. Blood samples were collected 1 week before transport in basal conditions, 1 week later immediately before loading, and after transport and unloading. After transport, donkeys showed significant increases in circulating T_4 ($P \leq 0.01$), fT_3 ($P \leq 0.001$), and fT_4 ($P \leq 0.01$) levels; while horses had significant increases in circulating T_3, fT_3 and fT_4 ($P \leq 0.01$) levels. Compared to donkeys' values, horses showed lower T_4 values in basal condition, before and after transport ($P \leq 0.001$); higher fT_3 values in basal condition and before ($P \leq 0.001$), and lower values ($P \leq 0.001$) after transport; higher fT_4 values ($P \leq 0.001$) in basal condition. The results indicate that short road transport of donkeys and horses induces the activation of the thyroid gland, with the same release of fT_3 and fT_4 iodothyronines, but with different preferential release of T_3 in horses and T_4 in donkeys after transport.

Variazioni delle iodotironine totali e libere negli equidi (Equus asinus ed Equus caballus) dopo un trasporto di breve durata

Parole chiave
Asino,
Cavallo,
Iodotironine libere,
Iodotironine totali,
Trasporto.

Riassunto
I cambiamenti delle iodotironine totali e libere sono stati studiati in 12 stalloni (6 *Equus asinus* e 6 *Equus caballus*), sottoposti a trasporto di breve durata su strada (50 km). I prelievi di sangue sono stati effettuati, in condizioni basali, immediatamente prima del carico e dopo il trasporto. I risultati ottenuti dopo il trasporto, rispetto alle condizioni basali e al pre-trasporto, hanno evidenziato aumenti significativi dei livelli di T_4 ($P \leq 0,01$), fT_3 ($P \leq 0,001$) and fT_4 ($P \leq 0,01$) negli asini, e un significativo aumento dei livelli di T_3, fT_3 and fT_4 ($P \leq 0,01$) nei cavalli. In particolare, rispetto ai valori rilevati negli asini, i cavalli hanno mostrato livelli di T_4 più bassi, in condizioni basali, prima e dopo trasporto ($P \leq 0,001$); livelli più elevati di fT_3 in condizioni basali e prima del trasporto ($P \leq 0,001$), più bassi ($P \leq 0,001$) dopo trasporto e, ancora, più elevati di fT_4 ($P \leq 0,001$) in condizioni basali. I risultati indicano che il trasporto breve su strada induce sia negli asini sia nei cavalli un'attivazione della tiroide, con una risposta sovrapponibile di fT_3 e fT_4, con un preferenziale rilascio di T_3 nei cavalli e di T_4 negli asini dopo trasporto.

Introduction

Thyroid hormones are commonly present in metabolic processes (Graves *et al.* 2002) and in thermogenesis mechanisms in domestic animals (Bird *et al.* 1998), including horses (Fazio *et al.* 2007, Fazio *et al.* 2008, Fazio *et al.* 2013) and donkeys (Fazio *et al.* 2012, Mendoza *et al.* 2013, Todini *et al.* 2010). Circulating thyroid hormones are important physiologic index of cellular thyroid activity, as the biological capacity of T_3 and T_4 can be used to cross the cellular membrane from the serum into the target cells. The mechanism of thyroid uptake into the cell and its driving force is probably also dependent on the concentration of the free hormones in the serum, even if physiologic stress could negatively affect thyroid hormone's transport (Hennemann *et al.* 2001).

Physiologic ranges of thyroid hormone reported for horses (Chen *et al.* 1981, Duckett *et al.* 1989), growing foals (Fazio *et al.* 2007), and donkeys (Elmansury *et al.* 2007) have a wide variability, because many intrinsic and extrinsic factors can influence the secretion of total and free iodothyronines, at the same time differences in the recorded variation may also be ascribed to differences in assessment techniques (Sojka *et al.* 1993).

Studies of the hormonal, metabolic, functional, and behavioural effects of transport have been provided in the extant literature focusing on horses (Medica *et al.* 2010, Munsters *et al.* 2013, Schmidt *et al.* 2010). Limited scientific data are available on the influence of transport on the horses' (Fazio *et al.* 2008, Fazio *et al.* 2009) and mules' (Greene *et al.* 2002, Greene *et al.* 2006) thyroid response, and no data are available for donkeys.

On the basis of the essential importance of the thyroid hormones in the regulation of metabolic processes, the present study evaluated the potential contribution of transport stress to the donkey's thyroid gland responses after short distance road transport, through assessing the changes in circulating total and free iodothyronines.

Materials and methods

All procedures, treatments, and animal care were in compliance with the guidelines of the Italian Minister of Health for animal experimentation[1], European Directives[2] and with the regulation on the protection of animals during transport and related operations[3].

The study was carried out on 6 healthy Ragusano stallion donkeys (aged 6.3 ± 0.8 years (mean ± standard deviation)) and 6 healthy Sanfratellano stallion horses (aged 5.8 ± 0.9 years), weighing 293 ± 5 kg and 560 ± 10 kg, respectively. Both horses and donkeys were transported from their stud farm to another breeding station. All subjects were transported over the same distance and via the same route. All subjects, used only for reproductive activity, had previous transport experience and familiarity with trailer and conspecifics. Hay and water were available before loading, but not during transport.

Blood samples (10 ml) were collected from each animal while it was in its box by jugular venipuncture using evacuated tubes without coagulant (Venoject, Terumo®; Leuven, Belgium), 1 week before loading and transportation, at 09.00 hours, in quiet conditions (basal-sample). Blood collection was repeated from each subject 1 week after the first collection, at 09.00 hours immediately before loading (before-sample), and immediately after transportation and unloading. Each blood sampling took few minutes. After unloading, animals were sampled, weighed and brought to their individual box and had feed and free drinking water from automatic waterers. They were treated in the same order by the same caretaker during all procedures, which were completed within 30 minutes. Individual live body weight (BW) was recorded at basal conditions, before and after transportation using large animal scales.

The commercial trailer used was 9.5 m long and 2.5 m wide, with a ceiling height of 2.5 m. Six compartments with swinging gates were available to separate animals. The donkeys' and horses' orientations were determined in relation to previous transport experience of each subject. In particular, per each load, 3 donkeys were placed in the trailer facing forward and 3 donkeys were placed facing backward. The same orientation was applied for the transportation of horses. Stocking density was about 2 m^2/subject. Rubber padding lined the sides of the trailers from the floor to an approximate height of 1.2 m. The animals were transported over exactly the same distance (50 km) and route (trunk road) in a commercial trailer for a period below 1 hour, by the same driver with high driving experience. During transport the truck had an average speed of 80 km/h. The journey took place in March. Temperature and relative humidity inside the trailer at the outset were 19.5°C and 62.4%, respectively, and, after a period of about 1 hour of transport, were 20.4°C and 63.5%, respectively. A hygrothermograph ST-50 (Sekonic Corporation, Tokyo, Japan) was placed near the middle of the trailer to monitor the temperature and the humidity continuously.

Serum total and free iodothyronine concentrations were analysed in duplicate using commercial

[1] Decreto Legislativo n.116, 27 Gennaio 1992. Attuazione della direttiva n.86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. *Off J* (Suppl), **40**, 18.02.1992.

[2] Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off J*, **L 358**, 18.12.1986 (Repealed by Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Off J*, **L 276**, 20.10.2010).

[3] Council Regulation (EC) n. 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97. *Off J*, **L 3**, 05.01.2005.

immunoenzymatic assays (EIA, RADIM, Rome, Italy). The method is based on a competitive EIA, using the procedure previously described, and the reagents were prepared as described in the manufacturer's protocol. Total (T_3, T_4) and free (fT_3, fT_4) iodothyronines in the sample competed with T_3, T_4, fT_3, and fT_4 conjugated with horseradish peroxidase (conjugate) for binding to specific antibody sites of anti-T_3, anti-T_4, anti-fT_3, and anti-fT_4 coated wells. At the end of the incubation, all unbound material was removed by aspiration and washing. The enzyme activity, which was bound to the solid phase, would be inversely proportional to the concentration of T_3, T_4, fT_3, and fT_4 in calibrators and samples, and was evidenced by incubating the wells with a chromogen solution (TMB) in substrate buffer. Colourimetric reading was performed using a spectrophotometer at 450 and 405 nm. 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.

Data are presented as mean values ± standard deviation (SD). To determine whether transport had any effect on total and free iodothyronine concentrations, a 1 way analysis of variance for repeated measures (RM-ANOVA) was applied. Significant differences between basal and before transport values, and between before and after transport values were established using Student's paired t-test.

Data were also analysed with two-way repeated measures (RM-ANOVA) for the comparison of orientations and species.

The level of significance was set at $P \leq 0.05$. All calculations were performed using the PRISM package (GraphPad Software Inc., San Diego, CA, US). The correlation between total and free iodothyronine concentrations was evaluated by linear regression (r), calculated using Pearson's method.

Results

Compared to blood values registered before transportation, donkeys displayed significant increases in circulating T_4, fT_3, and fT_4 levels ($P \leq 0.001$), but no for T_3 levels, after transport (Figure 1, Figure 2).

T_3 values of donkey ranged between 1.37 and 1.60 nmol/l; T_4 values ranged between 62.42 and 69.98 nmol/l; fT_3 values ranged between 2.44 and 8.39 pmol/l; fT_4 values ranged between 10.55 and 14.49 pmol/l. While RM-ANOVA showed a significant effect of transport for T_4 ($P \leq 0.01$), fT_3 ($P \leq 0.01$) and fT_4 ($P \leq 0.01$), no significant differences were observed between donkeys facing forward and those facing backward for thyroid hormones. Positive and significant correlations were observed in donkeys between T_3 and T_4 ($r = 0.939$; $P \leq 0.005$) and fT_3 and fT_4 ($r = 0.988$; $P \leq 0.0002$) before transport, and between T_3 and fT_3 ($r = 0.994$; $P \leq 0.0001$) and fT_3 and fT_4 ($r = 0.986$; $P \leq 0.0001$) after transport.

Compared to the blood values registered before transportation, horses displayed significant increases in circulating T_3, fT_3, and fT_4 levels ($P \leq 0.001$), but no for T_4 levels, after transport (Figure 1, Figure 2).

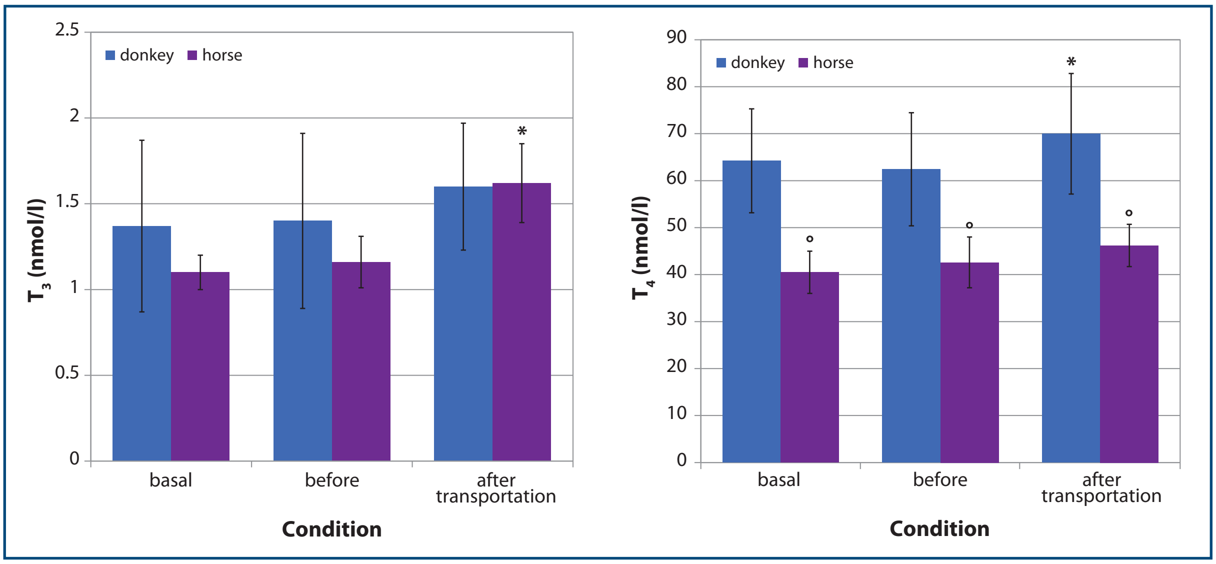

Figure 1. *Circulating total (T_3, T_4) levels (M ± SD) of donkey and horse in basal condition, before and after transport.* Asterisk indicates significant differences (*$P \leq 0.01$) in average hormone concentrations vs basal and before; symbol indicates significant differences (°$P \leq 0.001$) in average hormone concentrations vs donkey.

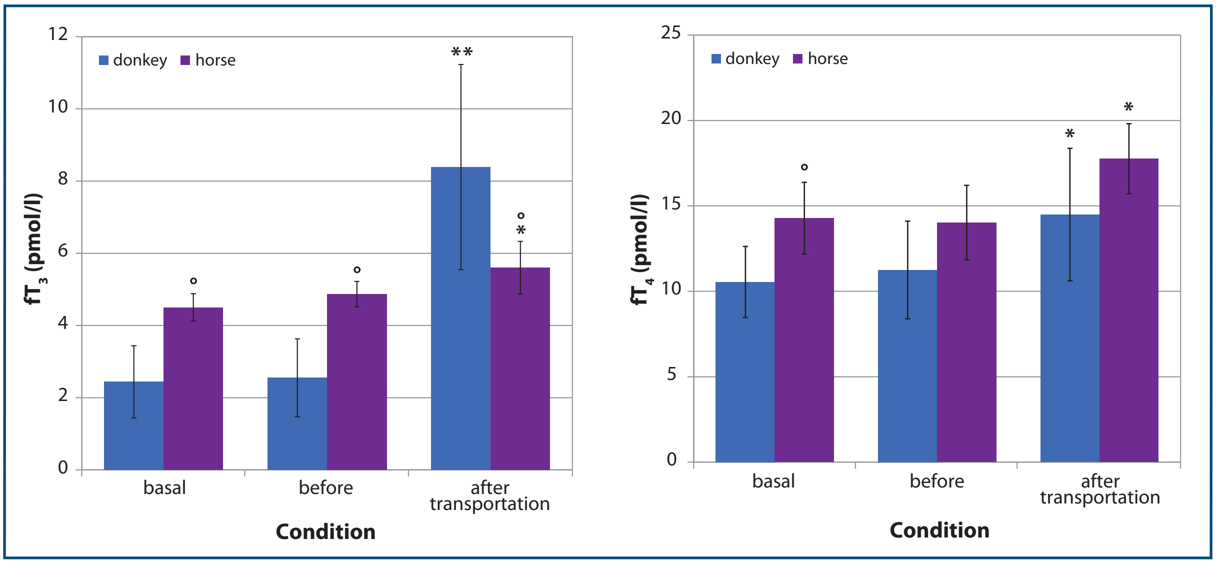

Figure 2. *Circulating free (fT_3, fT_4) iodothyronine levels (M ± SD) of donkey and horse in basal condition, before and after transport.* Asterisk indicates significant differences (*$P \leq 0.01$; **$P \leq 0.001$) in average hormone concentrations vs basal and before; symbol indicates significant differences (°$P \leq 0.001$) in average hormone concentrations vs donkey.

Horses' T_3 values ranged between 1.10 and 1.62 nmol/l; T_4 values ranged between 40.50 and 46.20 nmol/l; fT_3 values ranged between 4.50 and 5.60 pmol/l; fT_4 values ranged between 14.02 and 17.76 pmol/l. RM-ANOVA showed a significant effect of transport on T_3 ($P \leq 0.0001$), fT_3 ($P \leq 0.0001$), and fT_4 ($P \leq 0.0001$). No significant differences were observed for hormonal parameters between horses facing forward and those facing backward. Positive and significant correlations were observed in horses between T_3 and T_4 ($r = 0.859$; $P \leq 0.01$) and fT_3 and fT_4 ($r = 0.999$; $P \leq 0.0001$) before transport, and between T_4 and fT_4 ($r = 0.926$; $P \leq 0.005$) and fT_3 and fT_4 ($r = 0.998$; $P \leq 0.0001$) after transport.

Compared to donkey's values, horses showed lower T_4 values in basal condition, before and after transport ($P \leq 0.001$); higher fT_3 values in basal condition and before ($P \leq 0.001$), and lower values ($P \leq 0.001$) after transport; higher fT_4 values ($P \leq 0.001$) in basal condition.

No significant differences were observed between basal- and before-samples for total and free iodothyronines in either donkeys or horses.

On the basis of different orientation on the trailer, no significant differences were observed between total and free iodothyronines in animals facing forwards or backwards.

No significant decrease in BW after transport was observed.

Discussion

Data obtained in this study showed that circulating total and free iodothyronine levels reported in donkeys and horses are in agreement with those previously reported in horses (Cravana *et al.* 2010, Fazio *et al.* 2013, Ferlazzo *et al.* 2010), although T_4 levels in donkeys were the highest.

These results differed with our recent data on reference intervals of total and free iodothyronines of non lactating and lactating donkeys (Fazio *et al.* 2012, Mendoza *et al.* 2013, Todini *et al.* 2010) at least for circulating fT_4 and T_3 levels, that were the lowest in non lactating donkeys. On the contrary, circulating T_3 and T_4 levels of donkeys were higher than the range observed in healthy Sudanese donkeys (Elmansury *et al.* 2007). Some differences may also possibly be explained considering the influence of physiologic or other variables alongside to age (Chen *et al.* 1981), breed (Čebulj-Kadunc *et al.* 2003), gender (Fazio *et al.* 2007), diet (Pawell *et al.* 2000), management and stabling (Moons *et al.* 2005), sample collection, handling, and storage (Todini *et al.* 2010).

The influence of circadian rhythm on iodothyronine changes was not investigated because single measurements of these hormones are insufficient and inadequate to evaluate it.

Many endogenous and exogenous factors, i.e. environmental conditions and physiological variables, can affect or modify the course and amplitude of rhythms. Hence, although the existence of seasonal variations (Flisińska-Bojanowska *et al.* 1991) and daily rhythms (Duckett *et al.* 1989) of thyroid hormones was described in horses, data obtained exclude the possible influence of season and thyroid rhythms because blood samplings were performed at the same season and time.

These results show how the thyroid response actively modulates the reaction to short road transport, with a greater release of T_3 and T_4, in horses and donkeys, respectively, and with the same response for fT_3 and fT_4 levels. These results were supported by the existence of positive and significant correlations before transport between T_3 and T_4, and fT_3 and fT_4 in both donkeys and horses. The significant increase of T_4 levels in response to transport observed in donkeys could be either the result of preferential release of T_4 from the thyroid gland or of decreased peripheral monodeiodination of T_4. Although a concomitant no significant increase of T_3 levels was observed. These data partially confirm previous data obtained in horses after short road transport, with a significant increase of T_3, T_4, and fT_4 (Fazio *et al.* 2009).

In addition, the significant increases of free iodothyronines show their early and very active involvement in metabolic requirements in both donkeys and horses, as proved by the existence of positive and significant correlations between fT_3 and fT_4, both before and after transport. Additionally, the increases in total and free iodothyronines after transport confirm that thyroid hormones are markers of stress in domestic animals (Fazio and Ferlazzo 2003, Fazio *et al.* 2005, Fazio *et al.* 2007, Fazio *et al.* 2009).

The obtained data showed that transport of donkeys and horses influences thyroid responsiveness, with preferential release of total or free iodothyronines, suggesting that thyroid responses to short transport stress are influenced in similar modality in Equidae.

Furthermore, transport is inevitably associated with thermal stress, as confirmed by increase of temperature and relative humidity inside the trailer. However, the general increase of total and free iodothyronines after transport showed their involvement in the early stages of transport, within 50 km. In this study design, the animals were not previously acclimatized. However, evidence suggests that acute altitude exposure following transport stress in both horses and mules, produced significant increases in thyroxine, indicating that equids acutely acclimate within 2-3 days (Greene *et al.* 2002, Greene *et al.* 2006). In addition, energy costs of standing during transport were probably not considerable given to the quality and duration of transport. Moreover, short road transport could probably induce a metabolic contribution and the increases in total and free iodothyronines could be a thyroid contribution to energy requirement related to intermediary metabolism.

The data showed that transport stress seems to modify the thyroid response greatly, and previous experience of transport and the presence of co-specifics did not appear to influence it. The presence of the same staff for handling, loading, confinement, and unloading as well as the same veterinarian taking all blood samples did not reduce the response to short transport stress in these subjects, which were also already accustomed to transport.

In addition, the activity of thyroid gland to modulate total and free iodothyronines, however, remains unmodified in subjects independently of weather they were facing backward or forward. Hence, we conclude that the orientation does not influence thyroid hormone pattern in Equidae, confirming previous data reported in horses (Collins *et al.* 2000).

Thyroid hormone assessment after short transport has been shown to be effective in evaluating short term stress in donkeys and to provide an additional tool for distinguishing between total and free iodothyronine involvements, as reported in horses (Fazio *et al.* 2009).

These findings suggest that thyroid hormones of Equidae (*Equus asinus* and *Equus caballus*) play an important role in providing additional information for assessing transport conditions and related coping strategies to minimize the magnitude and duration of the stress responses.

The results of this study support our hypothesis that thyroid responsiveness to transport is similar but not equal between domestic donkeys and horses, as showed by the existence of positive and significant correlations between T_3 and fT_3 reported in donkeys, and between T_4 and fT_4 reported in horses after transport. Our hypothesis is also supported by published evidences related to reference values and differences between donkey and horse for hormonal, biochemical and haematological variables (Dugat *et al.* 2010).

We then conclude that transport is inevitably associated with a stress responses, which changes according to the inter-individual variability in both *Equus asinus* and *Equus caballus*. Stress responses were confirmed by higher standard deviation of total and free iodothyronines of both donkeys and horses.

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