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Effects of previous experience on total blood and free iodothyronine responses to isolation, restraint and shearing in sheep (*Ovis aries*)

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ABSTRACT: The aim of this study was to investigate a possible effect on thyroid hormone concentrations in peripheral blood in sheep submitted to isolation, restraint and shearing. Circulating T_3, T_4, fT_3 and fT_4 concentrations of No. 40 Pinzirita sheep were evaluated under baseline conditions, 24 h before isolation, restraint and shearing (T0), after isolation (T1), and after restraint and shearing (T2), with respect to the influence of a previous shearing experience (just shorn). A control group of 40 sheep was evaluated under baseline conditions (T0). Two-way RM ANOVA showed a significant effect of previous experience and sex on T_3 ($P \leq 0.05$) and fT_3 ($P \leq 0.01$) changes, with lower T_3 and higher fT_3 concentrations in just shorn than in intact females; on T_4 ($P \leq 0.005$), fT_3 ($P \leq 0.0001$) and fT_4 ($P \leq 0.001$) changes, with higher T_4, fT_3 and fT_4 changes in just shorn than in intact females; on T_4 ($P \leq 0.001$), fT_3 ($P \leq 0.0005$) and fT_4 ($P \leq 0.001$) changes, with higher T_4, fT_3 and fT_4 changes in intact females than males. The magnitude of total and free iodothyronine changes following handling practices suggests that restraint and shearing represent a moderate stressor in sheep, while isolation is probably perceived as a severe stress stimulus.

Keywords: sheep; isolation; restraint; shearing; total and free iodothyronines

Various management practices may cause differing degrees of stress in livestock, resulting in a wide range of endocrine responses (Mears et al. 1999; Carcangiu et al. 2008; Fazio et al. 2011; Fazio et al. 2014a; Fazio et al. 2014b). Stress can be engendered by physical facilities, human handling, climate and social and economic environment; when treatments are undertaken either for reasons of health maintenance (e.g. vaccination, dipping and drenching, marking, isolation, herding) or to attain production goals (e.g. shearing, tail docking, weaning and classing) (Hargreaves and Hutson 1997). Shearing is necessary for the well-being of sheep; nevertheless, it is also considered as a stress factor, capable of causing some behavioural changes (Avondo et al. 2000; Mousa-Balabel and Salama 2010). The effects of shearing on milk production and composition, body condition and lamb weight have been assessed (Corner et al. 2010; Leibovich et al. 2011; Sphor et al. 2011). Additionally, respiratory rate, heart rate and body temperature are widely used as physiological indicators for studying stress and adaptation to the environment (Mousa-Balabel and Salama 2010). In contrast, little is known regarding the involvement of thyroid hormones in response to stress (Fazio and Ferlazzo 2003; Ferlazzo et al. 2007) and whether or not any total or free iodothyronines can be used as objective indicators of stress in domestic animals (Fazio et al. 2007; Fazio et al. 2012; Fazio et al. 2014b). Plasma T_3 and T_4 concentrations were not affected by tail docking, castration, weaning, isolation and restraint in lambs (Mears and Brown 1997). The goal of this study was to investigate whether there is an effect on thyroid hormone concentrations in peripheral blood in sheep exposed to the physical and mental stress of isolation, restraint and shearing, by taking into account the influence of previous exposure to shearing.

MATERIAL AND METHODS

Animals, housing, feeding and care. The study was carried out on a flock of about two hundred

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Pinzirita breed sheep. The farm was located in Sicily, at latitude: 37°56'1''N, longitude: 13°39'58''E, 550 m above sea level. Forty sheep (20 males and 20 females) were evaluated under baseline conditions, 24 h before isolation, restraint and shearing (T0), after isolation (T1) and after restraint and shearing (T2). The influence of a previous shearing experience was taken into account. Shearing and the associated husbandry procedures lasted for an average of 5 min. Experimental sheep were penned separately from the rest of the flock in the sheep barn, 24 h before shearing, with visual, olfactory and acoustic contact with the flock. Shearing was done on one day. According to previous experience the experimental subjects were divided into two groups: group A: 20 sheep (10 males and 10 females), aged one to two years, without previous shearing experience (intact); and group B: 20 sheep (10 males and 10 females), aged older than two years, previously exposed to shearing (just shorn). A control group C of 40 sheep split into two subgroups represented by 10 intact males and 10 intact females, aged one to two years, and 10 males and 10 females just shorn, aged older than two years, was evaluated under baseline conditions (T0) and at the same time points (T1 and T2) as the experimental groups. During the day, the animals grazed on natural pasture, and were penned during the night, when they received a commercial concentrated food-supplement of 200 g per head of crude protein (CP) 20.4% plus metabolisable energy MJ (ME) 12.5/kg dry matter. The sheep also had free access to hay (CP 11.1% and 7.2 MJ ME/kg DM), and water was continuously available.

During the observational period, individual live body weights (BW_s) in kg were recorded using large animal scales, immediately after blood samplings.

The BW_s of males and females, aged one to two years, ranged from 40 to 43 kg, and from 37 kg to 39 kg, respectively. Sheep, older than two years, showed a BW ranging from 70 to 72 kg in males, and from 45 kg to 47 kg in females.

According to the traditional practices of Sicilian sheep breeding, the animals are usually shorn once a year, in late spring at the end of June.

The environmental temperature and humidity were recorded with a Hygrothermograph ST-50 (Sekonic Corporation, Tokyo, Japan). The weather was mild but wet; the average environmental temperature and mean relative humidity values on the experimental days were 28 °C and 60%, respectively.

The rectal temperature was measured with a clinical thermometer for 1 min and was recorded immediately before blood sampling.

Blood sampling. Sheep were accustomed to clinical routines and blood sampling; subjects were individually held in a corner of the pen, one at a time, and were easily restrained by the same handler assistant who held their halter during the collection of T0 and T1 samples. On the day of the experiment, post-restraint and post-shearing (T2) blood samples were obtained from animals in lateral recumbency, with feet tied using a lead rope.

All animals were sampled in the same order; thus, blood samples from control and experimental sheep were obtained at the same time of day for each group, via jugular venipuncture, to minimise the effect of circadian rhythms on thyroid hormones. Blood samples were collected 24 h before experimental practices (T0: 08:00 h), 1 h after isolation (T1) and after restraint and shearing (T2).

Laboratory analysis. Blood samples were kept at 4 °C until processing. Then, samples were centrifuged at 1500 × *g* for 15 min and the serum was harvested and stored in polystyrene tubes at –20 °C until total and free iodothyronines were determined. Hormone assays were analysed in duplicate using a commercially available immunoenzymatic kit (SEAC-RADIM, Pomezia, Rome, Italy). The respective intra- and interassay coefficients of variation 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; 6.6% and 9.6% for fT_4. The commercial kits were validated for total and free iodothyronines by establishing that dilutions of ovine serum resulted in curves identical to those obtained with the human standards supplied with the assay kits.

Statistical analysis. A one-way repeated measures analysis of variance (1-way RM ANOVA) was applied to test for any differences in the baseline values (T0) of the three different experimental groups, and for any differences in values after isolation (T1) and after restraint and shearing (T2). Results were analysed using repeated measures analysis of variance performed with software for statistical analysis (PRISM, GraphPad Software Inc., San Diego, CA, USA).

To determine the effects of livestock management procedures (isolation, restraint and shearing) by comparing differences in experience and sex, a two-way repeated measures analysis of variance (2-way RM ANOVA) was applied. When the F statistic was significant, the differences between baseline (T0)

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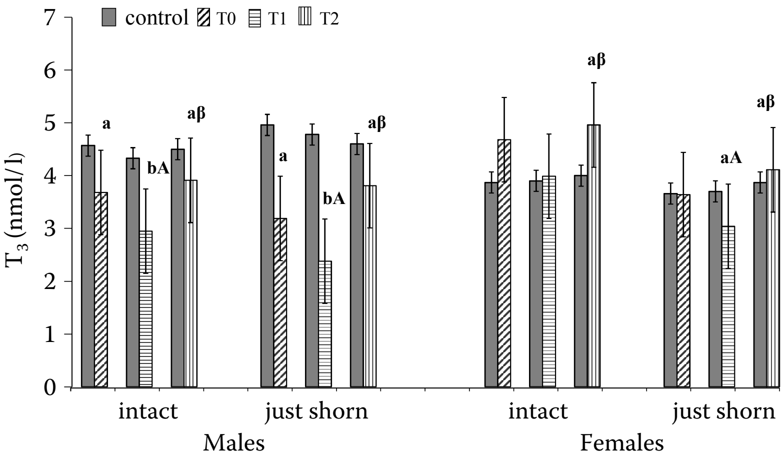

Figure 1. Total blood triiodothyronine levels (M ± S.D.) of sheep in the control group and in the experimental group before exposure to isolation, restraint and shearing (T0), after isolation (T1), after restraint and shearing (T2). Significant differences compared with control values ($^{a}P \leq 0.01$; $^{b}P \leq 0.001$); significant differences compared with T0 values ($^{A}P \leq 0.01$); significant differences compared with T1 values ($^{\alpha}P \leq 0.01$)

and post-livestock management practice (T1–T2) values were then assessed using a *post hoc* multiple comparison test (Bonferroni).

Linear regression and correlation between the different endocrine variables were also calculated. Statistical significance was set at $P \leq 0.05$.

RESULTS

Data obtained are presented as mean ± standard deviation (S.D.) for T_3 (Figure1), fT_3 (Figure 2), T_4 (Figure 3) and fT_4 (Figure 4).

Compared to the control group, males showed lower T_3 ($P \leq 0.01$) and fT_3 values at T0 (baseline values) and higher T_4; females showed higher T_4 and fT_4 ($P \leq 0.01$) values.

Compared to the control group, males showed lower T_3 ($P \leq 0.001$) and fT_3 values at T1 (after isolation values) and higher T_4; females showed lower T_3 (experienced: $P \leq 0.01$) and higher T_4 and fT_4 ($P \leq 0.01$) values.

Compared to the control group, males showed lower T_3 ($P \leq 0.01$) and higher fT_3 (intact: $P \leq 0.001$) values at T2 (after restraint and shearing), higher T_4 ($P \leq 0.01$) and higher fT_4 ($P \leq 0.01$) values; females showed higher T_3 ($P \leq 0.01$), fT_3 ($P \leq 0.001$), T_4 (intact: $P \leq 0.01$; just shorn: $P \leq 0.001$) and fT_4 ($P \leq 0.001$) values.

Compared to T0 values, males and females showed lower T_3 ($P \leq 0.01$) values at T1 (after isolation), higher T_4 ($P \leq 0.01$), fT_3 (males: $P \leq 0.01$; females: $P \leq 0.001$) and fT_4 ($P \leq 0.01$) values at T2 (after restraint and shearing).

Compared to T1 values (after isolation), males showed higher T_3 ($P \leq 0.01$), T_4 ($P \leq 0.05$), fT_3 ($P \leq 0.001$) and fT_4 ($P \leq 0.001$) values at T2.

One-way RM ANOVA showed a significant effect of livestock management procedures (isolation, restraint and shearing) on T_3 values of males (intact: $P \leq 0.01$; just shorn: $P \leq 0.0001$); on T_4 of males (intact: $P \leq 0.0001$; just shorn: $P \leq 0.005$), and females (intact: $P \leq 0.05$; just shorn: $P \leq 0.0001$); on fT_3 of males (intact: $P \leq 0.0001$; just shorn: $P \leq 0.0001$), and of females (intact: $P \leq 0.0001$; just shorn: $P \leq 0.0001$); on fT_4 of males (intact: $P \leq 0.0001$; just shorn: $P \leq 0.0001$), and of females (intact: $P \leq 0.05$; just shorn: $P \leq 0.0001$).

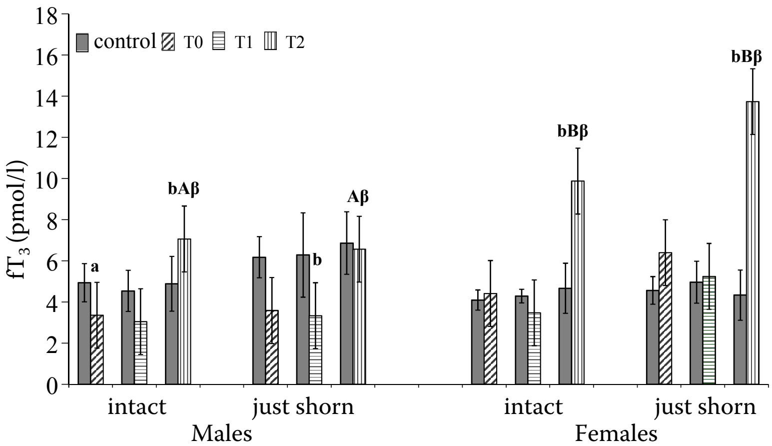

Figure 2. Free blood triiodothyronine levels (M ± S.D.) in sheep in the control group and in the experimental group before exposure to isolation, restraint and shearing (T0), after isolation (T1), after restraint and shearing (T2). Significant differences compared with control values ($^{a}P \leq 0.01$; $^{b}P \leq 0.001$); significant differences compared with T0 values ($^{A}P \leq 0.01$; $^{B}P \leq 0.001$); significant differences compared with T1 values ($^{\beta}P \leq 0.001$)

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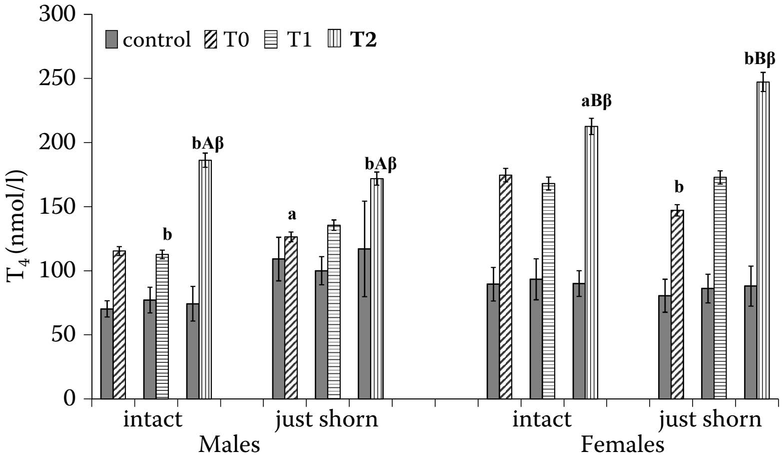

Figure 3. Total blood thyroxine levels (M ± S.D.) in sheep in the control group and in the experimental group before exposure to isolation, restraint and shearing (T0), after isolation (T1), after restraint and shearing (T2). Significant differences compared with control values ($^{a}P \leq 0.01$; $^{b}P \leq 0.001$); significant differences compared with T0 values ($^{A}P \leq 0.01$; $^{B}P \leq 0.001$); significant differences compared with T1 values ($^{\alpha}P \leq 0.01$)

Two-way RM ANOVA showed a significant effect of previous experience (just shorn) and sex on T_3 ($P \leq 0.05$) and fT_3 ($P \leq 0.01$) changes, with lower T_3 and higher fT_3 concentrations in just shorn than in intact females; on T_4 ($P \leq 0.005$), fT_3 ($P \leq 0.0001$) and fT_4 ($P \leq 0.001$) changes, with higher T_4, fT_3 and fT_4 in just shorn than in intact females; on T_4 ($P \leq 0.001$), fT_3 ($P \leq 0.0005$) and fT_4 ($P \leq 0.001$) changes, with higher T_4, fT_3 and fT_4 in intact females than in males.

Significant linear regressions and correlations (r^2) between T_3 and fT_3, fT_4 and fT_3,T_4 and fT_4 in just shorn and intact female and male lambs are shown in Table 1.

No significant differences between baseline values (39.2 ± 1.0 °C) and post isolation (39.1 ± 1.1 °C), restraint (39.2 ± 1.2 °C), and shearing values (39.3 ± 1.6 °C), in terms of rectal temperatures, were observed between males and females. Similarly, no differences were observed between the just shorn and intact animals. No significant linear regressions and correlations between total and free iodothyronines and rectal temperature were observed.

DISCUSSION

Comparison of our data related to total and free iodothyronines, with published data for ovine species (T_3: 0.96–2.30 nmol/l; T_4: 37.96–79.15 nmol/l; Reap et al. 1978; T_3: 1.50 ± 0.01 nmol/l; T_4: 100.38 ± 5.01 nmol/l; fT_3: 3.99 ± 0.36 pmol/l; fT_4: 24.71 ± 0.78 pmol/l; Anderson et al. 1988; T_3: 2.13 ± 0.07 nmol/l; T_4: 88.25 ± 1.74 nmol/l; fT_3: 4.68 ± 0.14 pmol/l; fT_4: 20.24 ± 0.47 pmol/l; Nazifi et al. 2008) revealed some discrepancies, although T_4 concentrations were in agreement with data observed in ewes (T_4: 152–218 nmol/l; Jawasreh et al. 2009), goats (T_4: 71–111 nmol/l; Todini 2007) and does (T_4: 78.50–104.89 nmol/l; Todini 2007). We speculate that the slight variations observed might be ascribed to differences in methods; in addition, some differences may also be explained by nutritional, physiological, environmental or geographic variables, related to different experimental conditions.

Compared to control group I, under baseline conditions (T0) lower T_3 and fT_3 and higher T_4

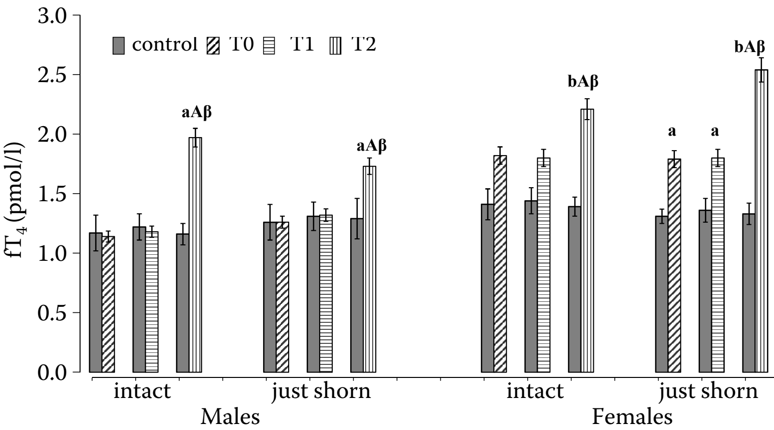

Figure 4. Free blood thyroxine levels (M ± S.D.) in sheep in the control group and in the experimental group before exposure to isolation, restraint and shearing (T0), after isolation (T1), after restraint and shearing (T2). Significant differences compared with control values ($^{a}P \leq 0.01$; $^{b}P \leq 0.001$); significant differences compared with T0 values ($^{A}P \leq 0.01$); significant differences compared with T1 values ($^{\beta}P \leq 0.001$)

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Table 1. Linear regressions and correlations between total and free iodothyronine concentrations in just shorn and intact female and male lambs

Thyroid hormones	Sex	Just shorn lambs		Intact lambs	
		r^2	P	r^2	P
T_3–fT_3	♀	0.26	0.005	0.18	0.01
	♂	0.35	0.0005	0.35	0.0005
fT_4–fT_3	♀	0.52	0.0001	0.31	0.001
	♂	0.35	0.0005	0.35	0.0005
T_4–fT_4	♀	0.27	0.005	0.42	0.0001
	♂	0.26	0.0005	0.26	0.0005

concentrations were observed in males, and higher T_4 and fT_4 concentrations in females, irrespective of previous experience. These data lead us to hypothesise that there exists a baseline response of thyroid hormones to general management practices, with active peripheral metabolism of T_3 and fT_3 only in males, and contemporaneously, T_4 reserves in both males and females. These findings also show that under baseline conditions thyroid concentrations are probably more affected by sex than by previous experience, especially considering that the T_4 changes were equal between males and females.

The T_3 decrease after isolation in just shorn and intact sheep, such as induced by confinement and weaning in lambs (Fazio et al. 2014b), and by food deprivation in stressed sheep (Wronska-Fortuna et al. 1993), could be interpreted as a rise in perceived isolation stimulus severity, with an increase in peripheral metabolism to meet metabolic requirements. Nevertheless, the proposed effect of short-term feed intake reduction and/or energy deprivation should also be taken into account to explain the steady and low T_3 values after isolation, as previously reported in sheep (Ekpe and Christopherson 2000; Abecia et al. 2001; Rae et al. 2002).

In addition, the higher T_3 concentration after restraint and shearing than after isolation in sheep confirms the onset of an earlier adaptation of the physiological stress response to handling which tends to decline over different stimuli (Fordham et al. 1989; Fordham et al. 1991; Hargreaves and Huston 1990). However, this is often slight and may depend on the nature and severity of the procedure (Jephcott et al. 1987; Smith and Dobson 2002). Therefore, it might be worthwhile to further investigate whether the T_3 changes might be related to some role in a coping strategy and arousal processes in response to stressful stimuli. In fact, the degree of brain arousal is known to co-vary with thyroid hormones, and a relationship between T_3 concentrations and cognitive activity has been found in humans (Tucker et al. 1984).

Additionally, the significant increases in T_4, fT_3 and fT_4 values after restraint and shearing showed that these practices were perceived differently than isolation, reflected, probably, in the high circulating levels of biologically active forms of free thyroid hormones. Highest are levels of T_4 synthesis, which represents the reserve form, and is an indicator of energy balance as previously reported in steers (Hayden et al. 1993). Moreover, the trend of T_4 values, with concentrations higher after restraint and shearing (T2) than at baseline (T0), independent of previous experience, and higher than post-isolation values (T1) only in just shorn males and females showed that previous shearing experience did not appear to influence T_4 values.

In addition, the superimposed trend of fT_3 and fT_4, with higher values after restraint and shearing (T2) than baseline (T0) and post-isolation values (T1) shows that previous shearing experience and sex did not appear to specifically influence changes in free iodothyronines. This hypothesis was confirmed by significant and positive correlations between fT_3 and fT_4 both in just shorn and intact males and females.

Finally, the increases in T_3, T_4, fT_3 and fT_4 following restraint and shearing could indicate a response to handling stress and suggest that sheep, independent of sex and previous experience, may successively cope with these stressful procedures.

The significant changes described for total iodothyronines after isolation, restraint and shearing were not in accordance with past studies that showed that T_4 and T_3 concentrations were not

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affected by tail docking, castration, weaning, isolation and restraint in lambs (Mears and Brown 1997). These findings confirm that the effect of a handling experience may depend on the age at which the handling occurs, and it may be possible to exploit the sensitivity of sheep to learning at certain ages; habituating sheep to handling routines, familiarising them with yard configurations and training leader sheep at critical learning periods may improve later handling (Hargreaves and Hutson 1997). The magnitude of total and free iodothyronine changes following handling practices suggests that restraint and shearing represented moderate stressors in sheep, while isolation was probably perceived as a severe stress stimulus (Hargreaves and Huston 1990; Al-Qarawi and Ali 2005). Moreover, the fact that T_3 values were lowest after isolation, with a significant increase after restraint and shearing both in just shorn and intact males and females, showed that the previous shearing exposure did not induce an earlier adaptation response to stress with habituation to the stimulus.

This is most likely due to the degree of habituation of the sheep to human presence and handling; in the present study, sheep, independent of previous experience, may not have perceived handling stimulus to be a stressful component of restraint and shearing. In contrast, isolation stimulus, in which the visual, chemical and acoustic communications with conspecifics were abolished, probably still resulted in physical and mental stress.

These data suggest that T_3 and T_4 contribute differently to homeostasis during livestock procedures, and confirm that their concentrations are a result of the balance between thyroid synthesis, secretion and peripheral metabolism. Likewise, both total and free iodothyronine changes confirmed that the levels of these hormones are balanced, according to the amount and affinity of binding protein.

Further evidence would therefore be required to completely explain the mechanisms of adaptation to stressful stimuli following handling practices and related correlations with the effects of total and free iodothyronines in the ovine species.

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