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Effect of multiple environmental stressors on the adaptive capability of Malpura rams based on physiological responses in a semi-arid tropical environment

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Running Head: Multiple stressor impact on sheep adaptation

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Abstract

A 45 day study was conducted where the primary objective was to evaluate the effect of simultaneously imposed multiple stressors (thermal, nutritional and walking) on the adaptive capability of Malpura rams based on changes in ingestive bahaviour, physiological, blood biochemical and endocrine responses. Twenty adult Malpura rams (average BW 44.9 Kg) were used in the study. The rams were divided into two groups, CON (n=10; Control) and MS (n=10; multiple stressors). All of the rams were stall fed with a diet consisting of 70% roughage and 30% concentrate. The CON rams were maintained in a shed under ambient conditions with *ad libitum* feeding without walking while MS rams were subjected to multiple stressors (thermal, nutritional and walking). The feed intake ($p<0.01$) was lower and water intake ($p<0.01$) was higher in MS compared to CON rams. Among the physiological measurements, respiration rate afternoon ($p<0.01$), pulse rate morning ($p<0.01$), rectal temperature afternoon ($p<0.01$), skin temperature afternoon ($p<0.05$) and both morning and afternoon scrotum temperature ($p<0.01$) increased significantly in MS rams. Both hemoglobin ($p<0.05$), packed cell volume ($p<0.01$), increased and plasma glucose ($p<0.01$) reduced significantly ($p<0.01$) in MS as compared to CON rams. Among the endocrine parameters, the plasma cortisol increased and plasma thyroxin reduced significantly ($p<0.05$) in MS rams. The study indicates that Malpura rams have the capability to adjust their physio-biochemical and endocrine responses to cope with multiple stressors in a hot-semi arid environment. Further the study also indicated that respiration rate, rectal temperature, scrotal temperature, hemoglobin, packed cell volume and cortisol may act as ideal biological markers for quantifying the impact of multiple stressors in Malpura rams.

Keywords: Adaptation, Cortisol, Heat stress, Malpura ram, Multiple stress, Nutritional stress, Respiration, Sheep, Walking stress

1. Introduction

Livestock production can be adversely affected by the detrimental effects of extreme climatic conditions. Climate change is considered to be the major threat to the viability and sustainability of livestock production systems in many regions of the world (Gaughan et al., 2009). A considerable population of poor people depends on animals for food, fiber, income, social status, security, and companionship. Hence, one of the biggest challenges currently facing animal science is the need to increase production in the context of climate change. The challenge is exacerbated because high production animals are subjected to greater influences by climatic factors, particularly those raised under tropical conditions (Martello et al., 2010).

High ambient temperature affects the ability of sheep to dissipate body heat, and as a result respiration rate, body temperature, heart beat, and water consumption increase (Marai et al., 2007). Increased body temperature and respiration rate are the major indicators of heat stress in sheep (Al-Haidary, 2004). Furthermore an increase in body temperature is usually associated with marked reductions in feed intake, redistribution in blood flow and changes to endocrine functions that will negatively affect the production and reproductive performance of sheep (Marai et al., 2007).

During stressful conditions various endocrine responses are invoked in an attempt to improve the biological fitness (reduce the impact of the stressor) of the individual. The front-line hormones to overcome stressful situations are glucocorticoids and thyroid hormones. The secretion of glucocorticoids is a classic endocrine response to stress (Kannan et al., 2000). Currently it appears that glucocorticosteroids provide an initial integrating signal which in conjunction with other hormones and paracrine secretions may determine specific behavioral, physiological and biochemical responses in an animal to allow some degree of adaptation when

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the animal is exposed to different environmental conditions **(**Wingfield and Kitaysky, 2002). The thyroid gland is one of the most sensitive organs of the ambient heat variation (Rasooli et al. 2004). The appropriate thyroid gland function and the activity of thyroid hormones are considered crucial if the productive performance in domestic animals is to be maintained (Todini, 2007). When the animals suffer due to the heat load, food ingestion is reduced and metabolism slows down, resulting in a hypo-function of the thyroid gland (McManus et al., 2009). Hence, measuring metabolic hormones such as thyroid hormones will give an indication of the mechanisms of adaptation.

Sheep in hot semi-arid environment are for the most part reared in extensive systems. The productive potential of sheep in these areas is influenced by their exposure to harsh climatic factors (Sejian et al., 2011). Sheep grazing in this ecological zone face extreme fluctuations in the quantity and quality of feed on offer year round (Martin et al., 2004). However, in the context of climate change, it is not only the heat stress that affects livestock. Other important environmental stressors include nutritional stress and walking stress which comes about due to the need to walk long distances to source adequate feed and water. Most of the productivity losses of livestock during the summer are incurred through low pasture availability (Sejian et al., 2013).

Therefore, from climate change perspectives, it is essential to study the influence of all the major environmental stresses simultaneously (Sejian et al., 2013) in order to understand in depth the adaptive capability of the target species, in this case sheep. Gaining this understanding may pave the way for identification of the ideal requirements for sheep to counteract such environmental extremes. Hence an attempt has been made in this study to determine the effect of multiple stressors that are simultaneously imposed on sheep. The primary objective of the study presented here was to evaluate the simultaneous impact of multiple environmental stressors

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(thermal, nutritional and walking) on the adaptive capability based on changes in ingestive bahaviour, physiology, blood biochemical and endocrine responses in Malpura rams.

2. Materials and Methods

This study was approved by the Central Sheep and Wool Research Institute animal ethics committee.

2.1. Site of study

The experiment was carried out at the Central Sheep and Wool Research Institute, which is located in the semi-arid region of India at longitude 75º 28´E, latitude of 26º 26´N at an altitude of 320 m above mean sea level. The average annual minimum and maximum ambient temperature ranges from 6 to 46 º C. The mean annual relative humidity (RH) ranges from 20 to 85%. The annual rainfall in this area ranges from 200 to 400 mm with an erratic distribution throughout the year.

2.2. Experimental design

Twenty adult Malpura rams (2 to 4 years old) with mean body weight of 44.9 ± 0.69 kg were used in a 45 day study. The Malpura is a triple purpose, hardy sheep breed, which originated in the arid and semi-arid areas of Western tropical India. The rams were divided into two groups, CON (n=10; control) and MS (n=10; multiple stresses). The animals were housed in asbestos-roofed, dirt floor sheds 2.4 m high at the center and 1.73 m high at the sides. The 3.66 × 7.32 m shed has a holding capacity for 100 adult rams. A wire mesh partition was used to hold the experimental animals at a stocking density of 3 m^2/animal. The four sides of the shed were covered with open-type wire mesh. The sheep were individually restrained while feeding in order to determine individual feed and water intake. Prior to the start of the experiment, these animals were acclimatized to the restraint. At 0900 h, both groups of rams were removed from the shed. The MS rams were subjected to heat and walking stress while CON rams were housed in a similar adjacent shed. The CON rams were maintained in the shed under ambient conditions (maximum ambient temperature exposed was 38 °C) for the 45 days of the study. The CON rams had *ad libitum* access to feed and water. The MS rams were subjected to multiple stressors each day: thermal, walking and nutritional. For 18 h of each day the MS rams were housed in the same shed as the CON rams. The first stressor was exposed to outdoor ambient conditions for 6 h each day (1000 h to 1600 h) where the temperature ranged from 39 to 44 °C. During this 6 h period MS animals did not have access to shade, feed or water. The second stressor was walking. The MS rams were subjected to walking stress by walking them for 14 km. This 14 km was covered in two spans. The animals took one hour and 30 minutes (1000 h to 1130 h) to complete one span (7 km) and the second span (7 km) was between 1400 h and 1530 h. A face mask was attached to the rams to prevent them grazing while walking. Prior to start of the experiment, the animals were acclimatized to these face masks (Sejian et al., 2012) in order to avoid any undue restraining stress. The third stressor was nutritional. Apart from the prevention of grazing while walking the MS rams were only fed at 30% of the *ad libitum* intake of the CON rams.

2.3. Feed sample analysis

All the rams were stall fed a diet consisting of 70% roughage and 30% concentrate. The composition of the diet was: roughage (*Cenchrus ciliaris*) and concentrate mixture (barley 65%, groundnut cake 32%, mineral mixture 3% and common salt 1%). Table 1 describes the dietary composition of the feed provided to the sheep. The crude protein (CP) of the feed sample was determined by Kjeldahl technique (AOAC, 1995). Neutral detergent fibre (NDF) was determined by the Van Soest et al. (1991) without sodium sulphite or amylase, whereas acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to method as described by Robertson and Van Soest (1981). The energy values of the diet are estimated as per the following formulae:

Total digestible nutrient (TDN) = digestible crude protein/kg + digestible carbohydrate/kg + 2.25 × digestible ether extract = 0.4 kg TDN where Digestible carbohydrate = (crude fibre + nitrogen free extract).

DE and ME are calculated as per the following formulae:

DE (MJ/kg)= 18.4096 × 0.4 = 7.36

ME (MJ/kg) =7.36 × 0.82 = 6.04

2.4. Climatic data

Table 2 describes the cardinal weather parameters during the study period. The minimum and maximum temperatures, dry and wet bulb temperatures and RH were measured using a digital thermo-hygrometer (Zeal, London, UK). The weather parameters were recorded twice daily at 0700 h and 1400 h.

2.5. Blood collection and plasma separation

Blood (5 mL) was collected at 1400 h on day 0, 15, 30 and 45 from each animal in the CON and MS treatment groups. Blood was collected from the jugular vein using 20 gauge sterilized needles and plastic syringe in tubes with heparin anticoagulant. Plasma was separated by centrifugation at 1870 g at room temperature for 20 minutes. The plasma was then divided into equal aliquots in microcentrifuge tubes, and kept frozen at -20°C until further analysis. Plasma samples were used to determine plasma glucose, cortisol, tri-iodo-thyronine (T_3) and thyroxin (T_4) concentration.

2.6. Variables studied

Feed intake (FI) and water intake (WI) were recorded daily. The following parameters were measured at 15 day intervals: respiration rate (RR), pulse rate (PR), rectal temperature (RT) skin temperature (ST), scrotum temperature (ScT), skin sweating rate (SSR), scrotum sweating rate (ScSR), hemoglobin (Hb) and packed cell volume (PCV). Physiological responses were recorded twice daily at 0800 h and 1400 h.

The RR (breaths/min) was recorded by counting flank movements/min with the help of a stop watch, from a distance of 4–5 m without disturbing the rams. The PR (beats/min) was measured by palpating the femoral artery. For recording the pulse rate, rams were restrained gently. The RT (^{o}C) was recorded using a clinical thermometer by inserting the thermometer by 6–7 cm inside the rectum inclined towards the wall of the rectum. RT was recorded by gently restraining the rams. The skin (flank region) and scrotum temperatures (^{o}C)were recorded using a non-contact infrared thermometer (B.S.K. Technologies, Hyderabad, India) by maintaining a distance of 5 to 15 cm. Aim the region where the temperature has to be taken, press the button on the device, the temperature is displayed immediately on the screen of the device. Sweating rate (SR) was recorded at weekly intervals at 1400 h. Sweating rate was measured by method as described by Berman (1957), based on the time taken for the chromatography paper disc impregnated with cobalt chloride to change color from violet to bright rose.

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The Hb and PCV were estimated using whole blood samples by methods as described by Balasubramaniam and Malathi (1992) and Jain (1986), respectively. Plasma glucose (Tietz, 1976) was estimated using Span diagnostic kits, India as per standard method using the UV–visible recording spectrophotometer (UV-160A; Shimadzu Corporation, Japan). Hormonal parameters such as cortisol (analytical sensitivity was 10 nM; the intra-assay and inter-assay coefficient of variations were 5.8 % and 9.2 %, respectively), thyroxin (T4) (analytical sensitivity 13 nmol/L; intra-assay and inter-assay coefficient of variations 5.1 % and 8.6 %, respectively) and tri-iodo-thyronine (T3) (analytical sensitivity 0.1 nmol/L; intra-assay and inter-assay coefficient of variations 3.3 % and 8.6 %, respectively) were estimated by RIA using gamma counter (PC– RIA MAS; Stretec, Germany) employing RIA kits supplied by Immunotech, Marseille Cedex, France.

2.7. Data analysis

The data were analyzed using the general linear model procedure by multivariate analysis of variance which included the effects of heat stress, nutritional stress and combined stressors and their interactions. Effect of fixed factors, namely treatment (TRT: Control (CON), and multiple stressors (MS) and days (DAY: longitudinal time over which experiment was carried out on days 0, 15, 30 and 45) and the TRT × DAY interaction of the variables studied were analyzed. Data was presented as mean ± SEM and statistical analysis was carried out using SPSS software, version 15.0. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Feed and water intake

The effects of multiple stressors on FI and WI are presented in Table 3. The FI was lower ($p<0.01$) in the MS rams compared with the CON rams on days 15, 30 and 45. Total FI was lower ($p<0.01$) in the MS rams (29.22 ± 0.36 DMI g/wk$^{0.75}$/day) compared with the CON rams (88.26 ± 0.56 DMI g/wk $^{0.75}$/day). However, WI was higher ($p<0.01$) in the MS rams (9.37 ± 0.17 L/DMI kg/day) compared with the CON rams (3.92 ± 0.06 L/DMI kg/day). Furthermore DAY influenced both FI and WI ($p<0.01$) indicating that the differences between TRT after day 0 persisted over time. In addition, there were significant TRT × DAY effects ($p<0.01$) on both FI and WI.

3.2. Physiological responses

The effects of multiple stressors on RR, PR and RT are presented in Table 4. Among the physiological responses, treatment influenced respiration rate afternoon (RRA) ($p<0.01$), pulse rate morning (PRM) ($p<0.01$) and rectal temperature afternoon (RTA) ($p<0.01$) between CON and MS groups. The RRA was higher ($p<0.01$) in the MS rams (66.1 ± 2.92 breaths/min) compared with the CON rams (58.1 ± 3.92 breaths/min). Similarly, RTA was higher ($p<0.01$) in the MS rams (39.1 ± 0.16 ºC) compared with the CON rams (38.6 ± 0.24 ºC). However, PRM was lower ($p<0.01$) in the MS rams (52.5 ± 2.15 beats/min) compared with the CON rams (58.1 ± 2.07 beats/min). Among the physiological responses, DAY influenced RRA ($p<0.01$), PRM ($p<0.01$), PRA ($p<0.01$) and RTA ($p<0.05$) indicating that the differences between TRT after day 0 persisted over time. However, TRT × DAY effects only influenced PRM ($p<0.01$).

The effects of multiple stressors on the skin and scrotum temperature and sweating rate are presented in Table 5. The treatment influenced skin temperature afternoon (STA) ($p<0.05$), scrotum temperature morning (ScTM) ($p<0.01$) and scrotum temperature afternoon (ScTA) ($p<0.01$) between CON and MS groups. The STA was higher ($p<0.05$) in the MS rams (39.0 ± 0.3 ºC) compared with the CON rams (38.5 ± 0.3 ºC). Similarly ScTA was higher ($p<0.01$) in the

MS rams (37.7 ± 0.2 ºC) compared with the CON rams (37.0 ± 0.2 ºC). However, the storm was lower ($p<0.01$) in the MS rams (33.6 ± 0.2 ºC) compared with the CON rams (34.1 ± 0.2 ºC). Furthermore, DAY influenced STM ($p<0.01$), STA ($p<0.01$), ScTM ($p<0.01$) and ScTA ($p<0.01$) indicating that the differences between TRT after day 0 persisted over time. However, TRT × DAY effects only influenced ($p<0.01$) STM and ScTM.

3.3. Blood biochemical and endocrine responses

The effects of multiple stressors on blood biochemical and endocrine responses are presented in Table 6. Except for T3, all blood biochemical and endocrine responses differed significantly between treatments. Among the blood biochemical responses, treatment influenced Hb ($p<0.05$), PCV ($p<0.01$) and plasma glucose ($p<0.01$) between CON and MS groups. The Hb was higher ($p<0.01$) in the MS rams (11.45 ± 0.53 g/dL) compared with the CON rams (10.42 ± 0.51 g/dL). Similarly PCV was higher ($p<0.01$) in the MS rams (33.19 ± 0.94 %) compared with the CON rams (28.74 ± 1.96 %). However, plasma glucose was lower ($p<0.01$) in the MS rams (42.45 ± 2.13 mg/dL) compared with the CON rams (51.86 ± 2.32 mg/dL). Among the endocrine responses, treatment influenced plasma cortisol ($p<0.05$) and plasma T4 ($p<0.05$) between CON and MS groups. The plasma cortisol was higher ($p<0.01$) in the MS rams (25.15 ± 2.53 nmol/L) compared with the CON rams (15.66 ± 2.57 nmol/L). However, plasma T4 was lower ($p<0.01$) in the MS rams (61.05 ± 7.16 nmol/L) compared with the CON rams (78.82 ± 7.52 nmol/L). Among the blood biochemical and endocrine responses, DAY influenced only the plasma glucose ($p<0.01$) indicating that the differences between TRT after day 0 persisted over time. However, TRT × DAY effects did not influence any of the blood biochemical or endocrine responses.

4. Discussion

The study presented here is of practical relevance as occurrences of multiple stressors are a common phenomenon in semi-arid tropical environments. Studying the effect of simultaneously imposed multiple stressors will provide relevant data that can be used to establish the impact of climate change on livestock production. The results of the current study have shown that multiple stressors influence feed intake, water intake, physiological responses, biochemical and endocrine responses differently when compared to individual stressors in the rams.

Feed and water intakes are the important parameters for establishing the adaptive capability of sheep (Minka and Ayo, 2009; Sejian et al., 2010a). Animals subjected to heat stress, attempt to adapt by reducing their feed intake and increasing their water intake. The significantly lower feed intake and higher water intake in the MS group shows the severity of these responses when sheep are exposed to multiple stressors. It is known that when sheep are exposed to high ambient temperatures their ability to dissipate body heat is reduced, and this results in an increase in respiration rate, body temperature and consumption of water, and a decline in feed intake (Marai et al., 2007). Marai et al. (2007) postulated a reason for reduced feed intake in sheep exposed to heat stress. They explained that exposure to high environmental temperatures, stimulates the peripheral thermal receptors to transmit suppressive nerve impulses to the appetite center in the hypothalamus, thereby causing a decrease in feed intake. The decrease in feed intake results in less metabolic heat production and this could be viewed as an adaptive mechanism. Several studies have stated that exposure of sheep to hot environmental conditions induces a marked increase in water turnover, as well as water intake (Monty et al., 1991; Ismail et al., 1995; Padua et al., 1997). An increase of water intake may occur as compensation for a deficit of body water which results from an increase of evaporation through the respiratory tract

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and the skin surface (Minka and Ayo, 2009; Darcan et al., 2008). Further, the significant influence of experimental days in the current study and the significant interaction between treatments and experimental days on both feed and water intake indicates the ability of these animals to adapt to the cumulative stressful conditions.

The physiological functions of animals, such as RT, RR and PR can favor its survival in a hot climate. Several researchers have studied physiological adaptation mechanisms such as RT, PR and RR in small ruminants (Otoikhian et al., 2009; Phulia et al., 2010; Sejian et al., 2014). In the current study the significantly higher RR and RT in MS group, when the animals were exposed to multiple stressors during the afternoon when the environmental temperature was in peak indicate the significance of these two parameters for adaptation in Malpura rams. Both RR and RT have been shown to be good indicators of thermal stress and may be used to assess the adversity of the thermal environment (Marai et al., 2002; Daramola et al., 2009). Onset and degree of thermal stress in an animal are best reflected by a rise in rectal temperature and respiratory dynamics, e.g. rapid, shallow respiration (Al-Haidary, 2004; Marai et al., 2007). The significantly lower PR during morning in the present study indicates the typical adaptive behavior of desert animals of keeping themselves cool during the night hours to cope up with the severe heat stress condition during the day time. Further the reduced pulse rate in the MS group may be due to a decrease in the metabolic rate as a result of restricted feeding of this group. This view was supported by the findings of several investigators who have reported that there is a correlation between heart rate and metabolic heat production (Yamamoto and Ogura, 1985; Barkai et al., 2002). Aharoni et al. (2003) suggested that heart rate decreases during thermal stress as a general effort by the animal to decrease heat production. This reduction could

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be achieved by the animal either by a reduction of feed intake, by a reduction in activity or both (Al-Haidary, 2004).

Skin temperature and scrotal temperature showed significant variation for treatment, indicating their significance for adaptation in sheep. Scrotal temperature was much more reliable in assessing the impact of multiple stressors than the skin temperature. This difference could be attributed to the wool coat on the body skin as compared to scrotal skin. Furthermore, the scrotum is an important thermoregulatory organ in sheep (Marai et al., 2007). Hence, scrotal temperature has higher significance for assessing the thermo-tolerant capability of sheep. Both skin and the scrotal sweating rate did not differ between treatments. This suggests that Malpura rams relied more on respiratory evaporative cooling mechanisms than the cutaneous evaporative mechanism. This finding was in contrast to the previous finding in Malpura ewes where there was a significant effect of multiple stressors on sweating rate (Sejian et al., 2013). Therefore, there may be a sex difference in adaptive mechanisms in Malpura sheep.

Multiple stressors significantly increased both Hb and PCV. The reason for this could be severe haemoconcentration as a result of the imposed multiple stressors. Both Hb and PCV are considered to be good indicators of stress in farm animals (McManus et al., 2009). Although the animals had *ad libitum* access to water the levels of Hb and PCV were higher in the multiple stressors group again highlighting the additive effects of multiple stressors. Generally during heat stress, severe dehydration has been reported in livestock, which ultimately leads to increased levels of Hb and PCV (Marai et al., 2007; McManus et al., 2009)**.** Further, severe water deprivation in the MS rams during walking stress could have aggravated the condition. The reduced plasma glucose in the MS treatment could be attributed to both food deprivations as well as the need to increase utilization of glucose during walking (Sejian et al., 2012). The decrease in glucose levels could be related to a decrease in insulin and thyroxine, which are closely associated with energy metabolism during stress (Rasooli et al., 2004). A decrease in plasma glucose could also be due to the marked dilution of blood or increase in the plasma glucose utilization to produce more energy for greater muscular expenditures required for high muscular activity (Rasooli et al., 2004; Sejian et al., 2010b). Nutrient restriction combined with increased glucose utilization due to increased respiratory muscular activity after thermal exposure, resulting in more reduction in glucose concentration in multiple stressors group.

Multiple stressors significantly increased plasma cortisol concentration, indicating that these animals are under stress. However, the cortisol concentration (25.15 nmol/L) obtained in this study was much lower than when two stressors (51.00 nmol/L) and three stressors (31.03 nmol/L) were simultaneously imposed on Malpura ewes (Sejian et al., 2010b; Sejian et al., 2013). This shows that Malpura rams were able to cope with the multiple stressors much better than Malpura ewes as reflected by minimum increase in plasma cortisol level to elicit the stress relieving effects as cortisol is thermogenic in nature which could contribute to additional heat load (Sejian et al., 2010b). Further the TRT × DAY also did not influence plasma cortisol level which was in contrast to the findings of two and three stressors simultaneously in Malpura ewes indicating that Malpura rams were able to cope up with the multiple stressors with the minimum possible increase in cortisol level. Similarly, the effect of multiple stressors on plasma T3 and T4 were not severe as only T4 differed between the groups. This was again being in contrast to the previous findings in Malpura ewes (Sejian et al., 2010b; Sejian et al., 2013) where much higher levels of both T3 and T4 were reported. This again points towards a sex difference in adaptive mechanisms in Malpura sheep. The reduced T4 concentration in multiple stressors group could be attributed to the reduced metabolic activity of these ewes to suppress heat production. During summer, the exposure of animals to high ambient temperature was associated with depression of thyroid activity, thereby causing a relatively lower concentration of thyroid hormones (Rasooli et al., 2004; Stockman et al., 2011). Besides endogenous and environmental, climatic factors, nutrition plays a primary role in thyroid gland activity and on blood thyroid hormone concentrations (Todini, 2007). These effects suggest that energy balance could play a major role in affecting the decrease in plasma thyroid hormone levels.

5. Conclusion

The findings from the current study have made a significant contribution to understanding the intricacies of multiple stressors on the physiological, blood biochemical and the endocrine responses of Malpura rams. The severities of multiple stressors were determined by the reduced feed and increased water intake in Malpura rams. Further, the results from the current study have shown that Malpura rams relied more on respiratory cooling mechanisms rather than cutaneous evaporative mechanisms to cope up to multiple stressors. In addition, the study indicates that Malpura rams have the capability to adjust their physio-biochemical and endocrine responses to cope with multiple stressors in the hot-semi arid environment. The study also indicated that RR, RT, ScT, Hb, PCV and cortisol may act as ideal biological markers for quantifying the impact of multiple stressors in Malpura rams.

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Conflict of Interest Statement

The authors declare that there is no any conflict of interest for this manuscript

Ethical Approval

This study was approved by the Central Sheep and Wool Research Institute animal ethics committee.

Authorship

The idea of paper was conceived and the experiment designed by V. Sejian. The experiment was performed by D. Kumar andV. Sejian.

The data were analysed by Dr. S. M. K. Naqvi. The paper was written by John Gaughan and V. Sejian.

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Table 1: The chemical composition, energy and nutrient contents of the diet provided to the animals

Nutrient Contents	**Roughage**	**Concentrate**
Ingredients	*Cenchrus ciliaris*	barley, 650 g/kg; groundnut cake, 320 g/kg; minerals 30 g/kg including 10 g/kg NaCl
Dry Matter (%)	92.7	93.3
Crude Protein (%)	8.4	14.0%
Ether Extract (%)	1.9	3.2
Neutral detergent Fibre (%)	72.6	48.5
Acid Detergent Fibre (%)	54.7	9.0
Acid Detergent Lignin (%)	20	6.23
Digestible Energy (MJ)	5.7	12.1
Metabolizable Energy (MJ)	4.7	10.1

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Table 2: Climatological data during study period both inside and outside the shed

Environment	Time of recording	Minimum temperature (°C)	Maximum Temperature (°C)	Dry bulb Temperature (°C)	Wet bulb Temperature (°C)	RH (%)
Inside the Shed	Morning (0800 h)	32.41±0.52	33.61±0.33	35.47±0.50	23.15±0.51	35.43±1.47
	Afternoon (1400 h)	36.50±0.50	38.25±0.22	42.57±0.45	24.30±0.42	33.14±1.72
Outside the Shed	Morning (0800 h)	34.33±0.30	35.12±0.37	32.02±0.38	24.45±0.56	40.60±2.36
	Afternoon (1400 h)	39.23±0.68	42.58±0.67	41.37±0.79	25.86±0.48	30.21±2.65

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Table 3: Effect of Multiple stresses on feed and water intake in Malpura rams

Attributes	Days	Treatments		Day Mean	Effects		
		CON	MS		TRT	DAY	TRT×DAY
Feed Intake (DMI g/w $^{0.75}$/day)	15	76.85 ± 0.54	29.26 ± 0.28	53.05 ±0.41	**	**	**
	30	92.32 ± 0.48	28.97 ± 0.43	60.65 ±0.46			
	45	95.61 ± 0.65	29.44 ± 0.38	62.52 ±0.52			
	Mean	88.26 ± 0.56	29.22 ± 0.36	58.74 ±0.46			
Water Intake (L/DMI kg/day)	15	4.72 ± 0.10	9.90 ±0.09	7.31 ± 0.10	**	**	**
	30	3.80 ± 0.05	10.03 ±0.18	6.92 ± 0.12			
	45	3.23 ± 0.04	8.18 ±0.24	5.71 ± 0.14			
	Mean	3.92 ± 0.06	9.37 ±0.17	6.65 ± 0.12			

CON-Control; MS-Multiple Stresses; TRT-Treatment; TRT x DAY - Treatment and Day interaction. * (P<0.05), ** (P<0.01), NS- Non-Significant

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Table 4: Effect of multiple stresses on respiration rate, pulse rate and rectal temperature in Malpura rams

Attributes	Days	Treatments		Day Mean	Effects		
		CON	MS		TRT	DAY	TRT×DAY
RRM (breaths/min)	0	24.0 ± 1.55	22.8 ± 1.84	23.4 ±1.70	**NS**	NS	NS
	15	27.6 ± 3.12	25.6 ± 1.97	26.6 ±2.55			
	30	25.7 ± 1.69	20.4 ± 1.51	23.1 ±1.60			
	45	22.6 ± 1.89	23.4 ± 1.89	23.0 ±1.89			
	Mean	25.0 ± 2.06	23.1 ± 1.80	24.1 ±1.93			
RRA (breaths/min)	0	56.2 ± 3.99	52.8 ±4.08	54.5 ± 4.04	**	**	NS
	15	57.4 ± 4.13	68.4 ±2.25	62.9 ± 3.19			
	30	66.2 ± 4.09	80.6 ±2.35	73.4 ± 3.22			
	45	52.6 ± 3.45	62.4 ±3.01	57.5 ± 3.23			
	Mean	58.1 ± 3.92	66.1 ±2.92	62.1 ± 3.42			
PRM (beats/min)	0	62.2 ± 1.65	63.2 ±1.79	62.7 ± 1.72	**	**	**
	15	60.8 ± 2.72	47.8 ±2.80	54.3 ± 2.76			
	30	54.4 ± 1.36	46.4 ±1.48	50.4 ± 1.42			
	45	54.8 ± 2.53	52.4 ±2.51	53.6 ± 2.52			
	Mean	58.1 ± 2.07	52.5 ±2.15	55.3 ± 2.11			
PRA (beats/min)	0	71.4 ± 1.40	68.6 ±1.61	70.0 ± 1.51	**NS**	**	NS
	15	60.8 ± 2.52	58.8 ±2.53	59.8 ± 2.53			
	30	62.4 ± 2.08	62.4 ±1.36	62.4 ± 1.72			
	45	60.4 ±3.29	58.4 ±2.17	59.4 ± 2.73			
	Mean	63.8 ± 2.32	62.1 ±1.92	62.9 ± 2.12			
RTM (ºC)	0	38.2 ± 0.13	38.0 ±0.13	38.1 ± 0.13	NS	NS	NS
	15	37.8 ± 0.23	38.0 ±0.25	37.9 ± 0.24			
	30	38.2 ± 0.25	38.0 ±0.28	38.2 ± 0.27			
	45	38.3 ± 0.34	38.1 ±0.18	38.2 ± 0.26			
	Mean	38.1 ± 0.24	38.0 ±0.21	38.1 ± 0.23			
RTA (ºC)	0	38.8 ± 0.22	38.9 ±0.13	38.8 ± 0.18	**	*	NS
	15	38.5 ± 0.20	39.1 ±0.19	38.8 ± 0.20			
	30	38.8 ± 0.24	39.3 ±0.17	39.1 ± 0.21			
	45	38.4 ± 0.31	39.1 ±0.15	38.7 ± 0.23			
	Mean	38.6 ± 0.24	39.1 ±0.16	38.9 ± 0.21			

RRM-Respiration Rate Morning, **RRA**- Respiration Rate Afternoon, **PRM**- Pulse Rate Morning, **PRA**- Pulse Rate Afternoon, **RTM**- Rectal Temperature Morning, **RTA**- Rectal Temperature Afternoon.

CON- Control; MS- Multiple Stresses; TRT-Treatment; TRT x DAY- Treatment and Day interaction

* (P<0.05), ** (P<0.01), NS- Non-Significant

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Table 5: Effect of multiple stresses on skin and scrotum temperature and sweating rate in Malpura rams

Attributes	Days	Treatments		Day Mean	Effects		
		CON	MS		TRT	DAY	TRT×DAY
STM (ºC)	0	35.0 ± 0.4	34.9 ± 0.2	34.9 ±0.3	**NS**	**	**
	15	35.7 ± 0.2	35.9 ± 0.2	35.8 ±0.2			
	30	38.4 ± 0.2	38.9 ± 0.1	38.7 ±0.2			
	45	37.5 ± 0.3	35.9 ± 0.2	36.7 ±0.3			
	Mean	36.7 ± 0.3	36.4 ± 0.2	36.5 ±0.3			
STA (ºC)	0	37.5 ± 0.4	37.2 ±0.3	37.4 ± 0.4	*	**	NS
	15	38.1 ± 0.4	38.8 ±0.2	38.5 ± 0.3			
	30	39.3 ± 0.3	40.3 ±0.3	39.8 ± 0.3			
	45	39.1 ± 0.2	39.6 ±0.2	39.3 ± 0.2			
	Mean	38.5 ± 0.3	39.0 ±0.3	38.7 ± 0.3			
ScTM (ºC)	0	33.7 ± 0.1	34.0 ±0.1	33.8 ± 0.1	**	**	**
	15	34.0 ± 0.1	34.2 ±0.1	34.2 ± 0.1			
	30	35.6 ± 0.2	35.0 ±0.3	35.3 ± 0.3			
	45	32.9 ± 0.3	31.3 ±0.4	32.1 ± 0.4			
	Mean	34.1 ± 0.2	33.6 ±0.2	33.8 ± 0.2			
ScTA (ºC)	0	39.1 ± 0.3	39.2 ±0.2	39.1 ± 0.3	**	**	NS
	15	38.2 ± 0.2	38.7 ±0.2	38.5 ± 0.2			
	30	36.8 ± 0.1	37.7 ±0.3	37.3 ± 0.2			
	45	34.1 ±0.2	35.1 ±0.2	34.6 ± 0.2			
	Mean	37.0 ± 0.2	37.7 ±0.2	37.4 ± 0.2			
SSR (g/m^2/hr)	0	132.9 ± 18.1	129.26 ±28.8	131.07 ± 23.5	NS	NS	NS
	15	156.0 ± 28.8	192.40 ±53.1	174.19 ± 40.9			
	30	141.1 ± 25.6	167.77 ±49.0	154.42 ± 37.3			
	45	80.8 ± 34.5	199.65 ±50.0	140.21 ± 42.3			
	Mean	127.68 ± 26.7	172.27 ±45.2	150.0 ± 36.0			
ScSR (g/m^2/hr)	0	926.0 ± 164.1	1190.0 ±176.4	1058.0 ± 170.3	NS	NS	NS
	15	1029.0 ±144.3	1327.0 ±162.3	1178.0 ± 153.3			
	30	975.4 ± 44.6	1136.0 ±60.9	1056.0 ± 52.75			
	45	1232.0 ± 163.2	1486.0 ±89.3	1359.0 ± 126.3			
	Mean	1040.0 ± 129.1	1285.0 ±122.2	1163.0 ± 125.7			

STM-Skin Temperature Morning, **STA**- Skin Temperature Afternoon, **ScTM**- Scrotum Temperature Morning, **ScTA**- Scrotum Temperature Afternoon, **SSR-** Skin Sweating Rate, **ScSR**- Scrotum Sweating Rate; CON- CON-Control; MS- Multiple Stresses; TRT-Treatment; TRT x DAY- Treatment and Day interaction
* (P<0.05), ** (P<0.01), NS- Non-Significant

Table 6: Effect of Multiple stresses on blood biochemical and endocrine responses in Malpura rams

Attributes	Days	Treatments		Day Mean	Effects		
		CON	MS		TRT	DAY	TRT×DAY
Hb (g/dL)	0	10.30 ± 0.39	10.33 ± 0.49	10.32 ±0.44	*	NS	NS
	15	11.10 ± 0.56	12.11 ± 0.86	11.60 ±0.71			
	30	10.01 ± 0.71	12.06 ± 0.38	11.03 ±0.55			
	45	10.28 ± 0.37	11.32 ± 0.37	10.80 ±0.37			
	Mean	10.42 ± 0.51	11.45 ± 0.53	10.94 ±0.52			
PCV (%)	0	32.00 ± 1.02	33.13 ±1.00	32.16 ± 1.01	**	NS	NS
	15	26.95 ± 1.98	31.78 ±1.44	29.36 ± 1.71			
	30	28.36 ± 3.07	33.65 ±0.68	31.00 ± 1.88			
	45	28.46 ± 1.75	34.20 ±0.63	31.33 ± 1.19			
	Mean	28.74 ± 1.96	33.19 ±0.94	30.97 ± 1.45			
Glucose (mg/dL)	0	51.42 ± 2.03	48.22 ±4.26	49.82 ± 3.15	**	**	NS
	15	54.12 ± 1.50	44.37±1.65	49.24 ± 1.58			
	30	49.02 ± 1.86	35.40 ±1.22	42.21 ± 1.54			
	45	52.86 ± 3.90	41.82 ±1.39	47.34 ± 2.65			
	Mean	51.86 ± 2.32	42.45 ±2.13	47.15 ± 2.23			
Cortisol (nmol/L)	0	14.19 ± 1.77	15.73 ±3.87	14.96 ± 2.82	*	NS	NS
	15	18.72 ± 2.02	26.43 ±2.17	22.57 ± 2.10			
	30	13.71 ±3.18	28.39 ±1.63	21.05 ± 2.41			
	45	16.02 ± 3.29	30.05 ±2.45	23.03 ± 2.87			
	Mean	15.66 ± 2.57	25.15 ±2.53	20.40 ± 2.55			
T3 (nmol/L)	0	1.27 ± 0.28	1.30 ±0.28	1.29 ± 0.28	NS	NS	NS
	15	1.52 ± 0.24	0.97 ±0.16	1.25 ± 0.20			
	30	1.62 ± 0.18	0.99 ±0.14	1.31 ± 0.16			
	45	1.46 ± 0.21	1.46 ±0.21	1.46 ± 0.21			
	Mean	1.47 ± 0.23	1.18 ±0.20	1.33 ± 0.22			
T4 (nmol/L)	0	79.81 ± 9.67	78.41 ±8.41	79.11 ± 9.04	*	NS	NS
	15	80.34 ± 5.62	54.16 ±9.00	67.25 ± 7.31			
	30	76.78 ± 6.91	47.64 ±6.31	62.21 ± 6.61			
	45	78.37 ± 7.89	63.99 ±4.93	71.18 ± 6.41			
	Mean	78.82 ± 7.52	61.05 ±7.16	69.94 ± 7.34			

Hb- Haemoglobin, **PCV**- Packed Cell Volume, T3- Tri-iodo-thyronine, T4- Thyroxin

CON- Control; MS- Multiple Stresses; TRT-Treatment; TRT x DAY- Treatment and Day interaction

* (P<0.05), ** (P<0.01), NS- Non-Significant

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Highlights

- Multiple stressors are a common phenomenon in many environments, and are likely to increase due to climate change.
- The severities of multiple stressors were established by the reduced feed and increased water intake in Malpura rams.
- Malpura rams relied more on respiratory evaporative cooling mechanisms than the cutaneous evaporative mechanism to counter multiple environmental stressors.
- The study indicated that respiration rate, rectal temperature, scrotal temperature, Hb, PCV and cortisol may act as ideal biological markers for quantifying the impact of multiple stressors in Malpura rams.

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