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TECHNICAL NOTE: APPLICABILITY OF INFRARED THERMOGRAPHY AS A NON INVASIVE MEASUREMENT OF STRESS IN RABBIT.

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Abstract: Among the main physiological stress indicators, the temperature evaluation is very important and innovative because it may be monitored without directly interacting with the animal. The use of a thermographic system, which is based on the detection of infrared radiation emitted by a subject, is a suitable method in order to measure temperature without any contact. In this research, a thermographic system was employed in order to single out the rabbit skin's zones most suitable for the temperature monitoring during stress challenges. Six hybrid rabbits were observed during induced stress; the areas selected as reference were: the ocular area (globe and periocular area), the internal auricle pavilion, and a shaved area of the head. The results of this pilot study show that the thermographic technique is a suitable method for the evaluation of temperature on rabbit. The best areas singled out were the eye bulb, the periocular area and the ear skin. The results concerning the effect of stress on cutaneous temperature showed that during stress condition a decrease in temperature occurs with respect to the basal condition ($\Delta T \simeq 1°C$) and this trend is more evident for the auricle pavillion. In fact, this reaction is more evidenced in the ear skin, where a vasoconstriction process occurs. Moreover, corticosterone levels slightly increase (P=0.08) following the stressor's challenge due to tonic immobility test. In this research, both temperature and the change in corticosterone level show that the stress reaction induced by tonic immobility test is stronger than the one due to the other stressors applied to rabbits.

Key words: Rabbit, measurement of stress, infrared thermography.

INTRODUCTION

Stress induced reactions in animals include behavioural and physiological modifications aiming at *coping* towards the stressor. As far as cardiovascular changes are concerned, stress may induce both modifications in heart rate and heart rate variability (HRV), as well as vascular changes in different body's parts, including the skin (Blessing, 2003; Walker and Carrive, 2003).

Similar physiological reactions have been shown stimuli in the rat's tail and in the rabbit's ear in response to alerting (Blessing and Seaman, 2003; Yu and Blessing, 2001). Emotional arousal during wakefulness may affect body temperature. Some authors already demonstrated hypothermia following physical restraint for example in mice, in adult rats and in rabbits (Grant, 1950). Franzini *et al.* (1981) found initially increased hypothalamic temperature in rabbits, which then decreased, after an emotional arousal due to the application of a Classical Aversive Conditioning Procedure. The contrary was shown in ear skin temperature, which initially decreased and then increased again. According to these authors emotional stress may directly affect effectors controllers for vasomotion. Complex regulatory mechanisms control body and skin temperature; these involve 'stimulation and withdrawal of sympathetic tone to arteries, arterioles, venules, veins and arteriovenous anastomoses' (Pollock *et al.*, 1994). Moreover, the cutaneous vasoconstriction contributes to the hyperthermia (Blessing

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and Seaman, 2003) in the body. Thus skin blood flow and sympathetic vasoconstrictor outflow to skin arteries affect skin temperature (Vianna and Carrive, 2005). The same vascular changes in response to fear eliciting stimuli in the rat have also been shown by Vianna and Carrive (2005), who found a marked drop in tail and paws temperature; on the contrary temperature of the eye, head and back increased. The functional hypothesis relies on the relationship between fear response and the increased arterial pressure and cardiac sympathetic output (Walker and Carrive, 2003). The main problems in measuring body and skin temperature are associated to the difficulty in recording them without inducing other stress reactions due to presence or handling by humans (Berz, 1998). Thus, infrared thermography may be used to remotely assess skin temperature, which is due to the infrared black-body emission (Jones and Plassmann, 2002), without interfering with the animal's behavioural reactions (Kastberger and Stachl, 2003). The thermographic technique in order to evaluate the stress response has been successfully applied in some research on domestic animals, although it has not yet been widely used in rabbits.

Thermographic techniques have been successfully tested in recent years also in other issues; for example, it was used in the evaluation of environmental stress on leaf transpiration Milazzo *et al.* 1994).

In a preliminary study on rabbit the research activity has been focused on defining reliable methods to measure rabbit's temperature variations by using an infrared camera, Avio TVS 700 microbolometric uncooled system operating in long wave range (8-14 microns). Zones of the rabbit skin most suitable for the temperature monitoring during stress reactions were singled out during this first tests (Luzi *et al.*, 2005). Variations of the observed temperature are in fact limited in a range of few degrees, so emissivity coefficient can be considered constant. Furthermore, the first study allowed detecting temperature variations according to different stress responses of the same subject, although variations due to individual metabolism were not taken into account. The selected areas as reference for the detection of the temperature changes were: the internal auricle pavilion, the eye and the periocular area.

Aim of this research was to obtain temperature measurements in the aforesaid reference areas, as well in a shaved area on the head, in order to measure the stress reaction due to different applied stressors in rabbits, measuring both the body and the peripheral temperature.

MATERIAL AND METHODS

Animals and treatments

The trial was assessed over a 2 months period at an experimental rabbitry. 6 hybrid rabbits were housed in a single cage (41×55×40 cm) at weaning (35 days of age) and fed *ad libitum* using a commercial standard diet. Water was also administered *ad libitum*.The average environmental temperature and relative humidity during the trial were constantly maintained at 21.2°C and 57.2% respectively, due to the climatic conditioning of the rabbitry. Each animal has been analysed in the baseline condition (Figure 1) and during the following individual tests: social stress, that consisted in changing the subject's cage and placing the subject together with another rabbit (two rabbits per cage) for 30 minutes; sudden noise stimulation, lasting 5 seconds; tonic immobility test: the rabbit was placed on the back and the maximum length of the test was three minutes. Each stressor was applied once.

Thermographic survey

In order to optimise the thermographic shots, rabbits have been transferred, in the first and second experimental phases, in special featured experimental cages (60×50×60 cm), structured with wire mesh spaced 5 cm. The first phase of the study has been considered as basal condition. The rabbits

Figure 1: Rabbits in four individual cages (basal conditions).

have been automatically monitored over 30 min., without stressing challenges, with a frequency of 1 image per minute. In the basal condition the rabbits were monitored putting the camera on a tripod 3 m far from the cages.

During the stress tests, 2 images (50 cm the first one, 20 cm the second one) have been recorded. Due to the presence of thick hair, we have analysed the ocular area (globe and periocular skin), the internal auricle pavilion, and a shaved area of the head (Figures 2-3). Temperature values were obtained as the mean value on homogeneous areas of 20-30 pixels. The error of each temperature was assumed as the standard deviation; this value was in general of the same order of the accuracy of the thermal camera (0.07°C).

Figure 2: Temperature distribution of a rabbit under laboratory conditions (eye, ear and shaved skin).

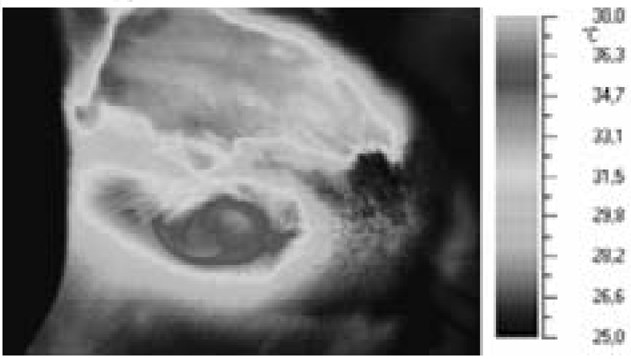

Figure 3: Detail of temperature distribution of a rabbit under laboratory conditions (eye and shaved skin).

Eyes emissivity has been supposed equal to the water emissivity. The skin and fur emissivity has been supposed equal to 0.97 in the 8-14 µm wave-length range (Pajani, 1989).

Variations due to individual metabolism were not taken into account, because the time interval between stress stimuli and thermographic shoot is shorter than the typical time of change of temperature in skin due to water holding capacity.

Physiological Measurements

In order to analyse corticosterone level variation following the stress induction, two blood samples have been taken from each experimental rabbit immediately before and 30’ after the tonic immobility test. The blood has been collected using an insulin syringe and subsequently the serum was analysed through a specific commercial kit for mouse and rat (I-125 MP Diagnostic Division®) based on RIA competition method with double antibodies. The methodology was modified for rabbit, using a pre-dilution of serum 1:30; the analyses were made in duplicated. The samples were processed by a gamma counter (Perkin Elmer®).

Statistical analysis

Infrared thermography data

Thermographic data, divided per shoot areas (eye/ear) are average obtained on 6 rabbits considering 5 days of measurement. The value of each subject represents the average of thermographic images video-recorded in the same experimental conditions. Shoots were recorded in the following sequence for each rabbit (Table 1)

Corticosterone level

Corticosterone data have been analysed using a variance analyse according to a general linear model (PROC GLM, SAS, 1999). Hormonal values were treated as continuous variables and the fixed effects were the blood sampling (basal and stress conditions), the interaction between the blood sampling and the basal or stress conditions and the standard error of the mean.

RESULTS AND DISCUSSION

The results of the preliminary test on rabbit A, taken as an example in order to define the best area to record with termography, are shown in Table 2.

Due to the low spatial resolution of the thermal image (5 pixel per cm) at the shoot distance in the baseline condition it was difficult to distinguish between periocular area and eye. In the periocular area the presence of fur gives the higher error because of the average occurring among warm skin pixels and cold fur pixels. In the further analyses periocular area was not considered at all.

The temperature of the shaved area of the head could be measured but, in this position, the main problem was the presence of the fur during the subsequent tests. In order to avoid repeated shaving of the animal, possibly acting as another uncontrolled stressor due to handling, also this area of measurement could not more be used (Figure 3).

Table 1: Shoots recording sequence for each rabbit.

Condition		Images recorded
baseline condition	30 min	30
social stress (2 rabbit per cage)	30 min	1 (after 20 minutes)
noise stimulation	5 s	1-2 from far and from narrow
tonic immobility	maximum 180 s	1-4 depending on duration of the immobility

Table 2: Temperature valued by infrared termography in different body positions.

Rabbit A (Basal condition)	Bulb and pupil	Periocular area	Internal auricle pavilion	Shaved area of head (Average T^a)
Average T^a± S.E.	35.6 ± 0.5	33.5 ± 0.85	36.5 ± 0.9	33.6 ± 0.6

S.E.: standard error

In Figure 4, the temperatures *vs.* time evolution of the ear pavilion and of the eye are reported, for the six rabbits during the first 30 minutes after been moved in the cage for the basal phase. In the first minutes rabbits were exposed to the stress due to the change of the cage and the presence of the operator.

The data show that temperature is quite constant after about five minutes, time interval in which rabbits could be affected by the adaptation period to the new cage**.** During the following period the ear temperature progressively increases compared to eye temperature (till 1.5°C); on the contrary, the temperature of the eye remains uniform. The difference of temperature between eye and ear was higher than the standard deviation of the data. On the contrary, rabbits show a drop in ear average temperature between the basal and stress conditions. In particular the different temperature between ear and eye ($\Delta T = T_{ea} - T_{ey}$). Over the six rabbits is positive in the basal condition and negative in the different stress conditions. The data are presented in the Figures 5 and 6 divided per area of analysis. A reduction of the ear temperature according to the different stressors is shown (Figure 5). On the contrary, the eye temperature shows more constant values (Figure 6). The stress response to tonic immobility seems to be higher than the ones to the other stressors. This may be due to the fact that the former stressor implies that the animal is restrained, while during social and noise stress the rabbit is free to move in the cage.

The results indicate that the best site to check an eventual stress condition is the ear skin, due the vasoconstriction process, as showed in a previous work by Vianna and Carrive (2005). These authors used the infrared thermography to check eventual changes in skin temperature during a conditioned fear response to context in rats. A marked drop in tail and paw temperature was observed in fear conditioned rats. In contrast, temperature of the eye, head and back increased, thus showing that fear evokes a skin vasoconstriction in the tail and paws. It could be a preparatory response to a possible fight and flight reaction to reduce blood loss in the most exposed parts of the rat's body in case of injury. During the acute stress response to fear eliciting stimuli, i.e. the flight or fight reaction

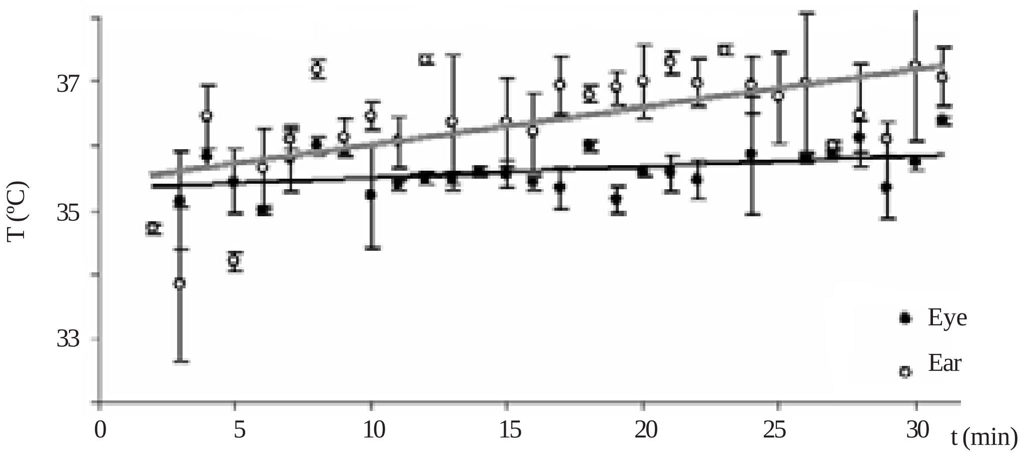

Figure 4: Temperature evolution during the basal conditions (average on 6 rabbits).

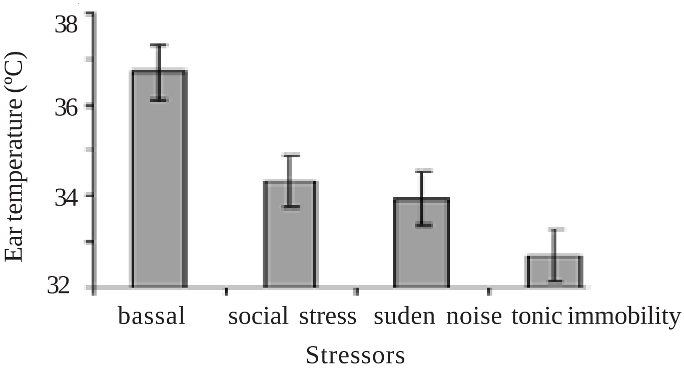

Figure 5: Ear temperature according to the different stressors and basal conditions plus standard deviation.

(Henry and Stephens, 1977), a reduced blood outflow to the body extremities, such as ear and tail, may lower the possibility to lose blood. In fact these ones are more likely to be injured in the case of a fight (Blessing, 2003). The ear vasoconstriction depends also on the adaptive *coping* strategies evolved by the species. Moreover, in our study, corticosterone levels slightly increased (*P*=0.08) after the stressors challenge (47.80 and 80.16 ng/ml for basal and stress condition, respectively). The increased levels of corticosterone following the stressor challenge agree with the hypothesis of the HPA activation during the stress response. Corticosterone levels as well as modifications in EEG, hyppocampal rhythmic activity (Fontani *et al.*, 1982) and cerebral glucose utilization (Passero *et al.*, 1981) have been correlated to the reaction in tonic immobility test (Carli, 1982) which has been used to test the rabbits' reactivity also according to handling (Verga *et al.*, 2004). Corticosterone levels are higher in subjects more susceptible to the test, thus suggesting a positive correlation between animals' reaction, i.e. the duration of the immobility, and the neuro-endocrine adrenocortical axis (Carli *et al.*, 1979).

CONCLUSIONS

The results of this pilot study show that the thermographic technique is a suitable non-invasive method for the evaluation of temperature variation in rabbit, revealing the temperature variations according to different applied stressors. In fact the ear temperature decreases following the stress challenges, mainly in the tonic immobility test, thus suggesting a vasoconstriction effect. At the same time, the HPA axis activation due to the stressor application is shown by the significant increase of the corticosterone level.

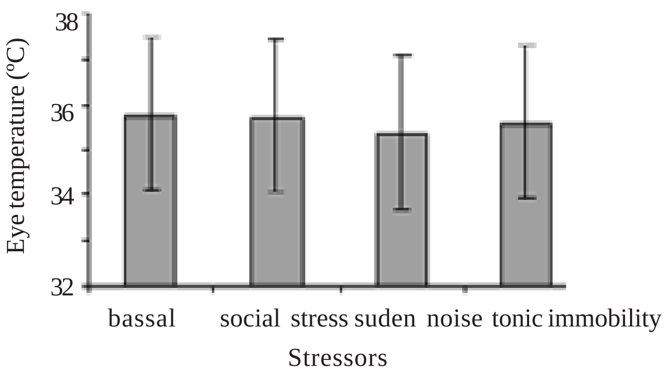

Figure 6: Eye temperature according to the different stressors and basal conditions plus standard deviation.

Further research is needed in order to obtain more results, measuring also the periocular area temperature, increasing the number of animal and correlating to the body temperature in order to standardise the methodology.

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