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The Relationship between Heat Stress, Survivability and Blood Composition of the Domestic Chicken[1])

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Summary: In order to better understand the metabolic changes leading to death which take place in the chicken during acute heat stress, the blood composition was determined in surviving and non-surviving chickens. The following blood analytes were determined: glucose, uric acid, serum total proteins, inorganic phosphate, total and ionized calcium, sodium, potassium, triiodothyronine, thyroxine. The haematocrit, erythrocyte creatine kinase (total and the isoenzymes) and haemoglobin fractions were also measured. Blood was taken from the wing vein before and after heat stress.

Eight-week-old "Anak 2000" broilers were kept in a climate chamber at 24 °C/40% relative humidity during a 14-hour day, and at 20 °C/40% relative humidity during a 10-hour night. The birds were subjected to heat stress by exposing them to 40 °C/30% relative humidity for 3 hours.

Significant differences between heat-stressed surviving and non-surviving chickens were seen in the blood levels of glucose, uric acid, total and ionized calcium, potassium, triiodothyronine, erythrocyte creatine kinase (total and isoenzymes). Differences were also seen in the levels and ratio of the 2 haemoglobin fractions.

The significance of these changes, and their potential use as markers for heat resistance is discussed.

Introduction

Permanent and acute heat exposure are among the stress factors affecting the metabolism of men and animals. Ambient temperatures exceeding the thermoneutral range lead to an elevated core temperature, leading to a number of responses, which result in the neutralisation of metabolic changes on one hand, and reduction of the body temperature on the other hand (1–4).

Tissue response to hyperthermia is associated with injury to the vascular endothelium and consequent altered vascular permeability and oedema. An increase in the body temperature of any living creature concomitantly increases its metabolic rate. Above a certain thermal limit, denaturation of enzymes and precipitation of other proteins occur. In addition, "melting" of the lipid bilayers of cell membranes takes place, leading to changes in membrane characteristics and permeability, associated with leakage and loss of cellular constituents (5, 6).

Hyperthermia is manifested by a disturbance of the heat regulating mechanism of the body. Prolonged exposure to high environmental temperature causes peripheral blood vessels to dilate. Heat cramps may ensue, due to the subsequent loss of salt and deranged electrolyte balance (7–13).

Among the clinical signs seen during hyperthermia are weakness, muscle tremors and collapse (11).

In the chicken, which lacks sweat glands, one adaptational response for cooling the body is panting, i. e. accelerating the breathing frequency. This leads to polypnea and a higher gas exchange rate, resulting in loss of carbon dioxide and enrichment of oxygen in the blood, thereby worsening the blood gas balance and leading to the development of respiratory alkalosis (1, 8, 14).

Men and animals can adapt to varying concentrations of oxygen and carbon dioxide by the change of haemoglobin affinity for these gases. This affinity change is accomplished by a change in concentration of phosphorylated intermediates such as 2,3-bisphosphoglycerate or inositol phosphate, which complex with haemoglobin, leading to higher or lower binding affinity of oxygen and carbon dioxide (6, 15, 16).

Animals behave and respond differently to heat stress. Furthermore, their structural composition and genetic characteristics contribute to their ability to resist heat stress. Knowing the factors that contribute to increased resistance is of great importance for understanding the pathological processes leading to death, and these factors are also possible markers for the determination and selection of heat-resistant animals.

Using clinical chemistry technology, the evaluation of blood composition can be a useful method for detecting

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changes due to heat stress. Blood composition changes as a result of metabolism, nutrition and cellular damage. Thus, blood levels of metabolites such as glucose, uric acid, minerals and proteins will be greatly affected by metabolism, nutrition and organ function. These changes can be measured by blood analysis and are related to the altered metabolism (15, 17).

It has been shown previously that a correlation exists between the degree of feathering of birds and the response to heat stress (18, 19), and that naked-neck chickens are more resistant to heat stress.

Even in chickens of the same age, size and breed, large variations were observed in their responses to heat stress, as evaluated by blood composition and behaviour. These differences suggest that genetic factors may contribute to heat resistance in chickens. Since these genetic factors are expressed in the biochemistry and metabolism of the organism, it should be possible to identify quantities affected by such factors, and use them to improve our understanding of the sequence of events leading to death from heat stress. Furthermore, we might use some of these quantities for evaluating and predicting the resistance of individual animals to heat stress, and by selecting such animals to produce a line of chickens with increased resistance to heat.

The objectives of the present study were to evaluate the biochemical changes associated with heat stress in chickens and their characteristics in surviving and non-surviving animals.

Materials and Methods

Broilers

One hundred and fifty one-day-old Anak 2000 broilers were first grown in a brooder house for 5 weeks, after which they were transferred to cages, one bird per cage until the age of eight weeks (mean 2.50 kg in weight). Both males and females were used. One week prior to the experiment, the broilers were transferred to the climatic chamber and kept at 24 °C/40% humidity during a day of 14 hours duration, and at 20 °C/40% humidity during a night of 10 hours duration, for acclimatisation to the new environmental conditions. Food and water were given ad-libitum, and the quantities consumed were measured for each individual daily.

Heat Shock

The chickens were exposed to high environmental temperatures to cause acute heat shock. The temperature was gradually increased to 40 °C/40% humidity during a period of two hours. The broilers were then kept for three hours under these conditions, after which the temperature was reduced to 25 °C/40% relative humidity. Following heat shock, the birds were divided into two groups:

(1) those surviving the heat shock and

(2) those not surviving the heat shock and dying within 24 hour after the heat shock.

Rectal temperatures were measured by a telethermometer model 46 TUC (Yellow Spring Instruments Co. Inc.).

Blood withdrawal and analysis

Blood samples were taken twice, prior to heat shock and during heat shock, three hours after reaching the target temperature. The blood was taken from the wing vein into two tubes, one for whole blood, containing lithium-heparin, and the other for serum. The heparinised blood was immediately put on ice and transferred to the laboratory. The non-heparinised blood was allowed to clot and the serum was separated by centrifugation at 600 g for 10 minutes.

Blood and serum analysis

The following analytes were determined in the serum: inorganic phosphorus (20), uric acid (21), total calcium (22), ionized calcium (23), potassium and sodium (23), total proteins (24), triiodothyronine (T_3) and thyroxine (T_4) using radioimmunoassay (Serono, Switzerland).

Whole blood was centrifuged at 600 g, for 10 min to separate the plasma, and the erythrocytes were washed twice with saline by their resuspension and centrifugation in the cold. In the plasma, glucose was determined enzymatically (25). The erythrocytes were haemolysed in water, the membranes were precipitated by centrifugation at 10 000 *g* for 10 minutes, and the supernatant was used for determination of haemoglobin (5), and total and isoenzyme levels of creatine kinase (ATP : creatine N phosphotransferase EC 2.7.3.2). Creatine kinase was measured spectrophotometrically (26); the isoenzymes (MM, MB, BB) were separated by electrophoresis (Helena Lab. U. S. A.) and their relative distribution, expressed in % of total activity, was determined using a "Cliniscan II" densitometer (Helena Lab. U. S. A.).

Chemical and biochemical determinations were performed on a "Kone-Progress" (Finland) autoanalyser.

Blood haematocrit was determined following centrifugation.

Haemoglobin was separated by electrophoresis using Helena's kit and according to the manufacturer's instructions (Helena Lab. U. S. A.). The various fractions were quantitated using a "Cliniscan II" densitometer.

Statistics

Means, standard deviation, correlation coefficient and significance, were determined using the general linear model (GLM) procedure of the SAS program (27).

Results

Changes in the blood levels of various analytes, following heat stress, are given in table 1. There were significant changes in blood analyte levels in both surviving and non-surviving groups. The changes can be divided into two: those showing similar patterns and those showing different patterns between the surviving and non-surviving groups.

Analytes changing in the same direction in both groups were inorganic phosphorus, total calcium and triiodothyronine (decreasing), and sodium, thyroxine and erythrocyte creatine kinase, isoenzymes BB and MB (increasing) and water consumption. Analytes changing in the opposite direction in the two groups were glucose, uric acid and erythrocyte total creatine kinase, ionized calcium, potassium and haemoglobin fractions.

The blood levels of glucose, uric acid, triiodothyronine and thyroxine in surviving and non-surviving chickens before and after heat stress are shown in table 2. In the non-surviving group the changes of serum levels of glu-

Tab. 1 Pattern of changes and their significance following heat stress.

Group	Serum										Erythrocyte creatine kinase				Hb-1/Hb-2	PCV	Water consumption
	Glucose	Uric acid	Total protein	Phosphate	Ca Total	Ca^{2+}	K^+	Na^+	T3	T4	Total	BB	MB	MM			
Survivor	↑*	↓*	=	↓*	↓*	=	=	↑*	*	↑	↑	↑	↑	↓	↓	=	↑*
Non-survivor	↓*	↑*	=	↓*	↓*	↓*	↑*	↑*	↓*	↑*	↓*	↑	↑*	↓*	↑*	=	↑*

* Significantly different from 0 time; $p < 0.01$.
PCV = Packed Corpuscular Volume, haematocrit.

Tab. 2 Blood levels of glucose, uric acid, T3 and T4 in the blood of surviving and non-surviving broilers before and during heat stress.

	Analyte							
	Glucose (mmol/l)		Uric acid (µmol/l)		T3 (nmol/l)		T4 (nmol/l)	
	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors
Before heat stress								
$\bar{x}$	12.71	12.77	397	359	3.72	4.36	86	92
SE	0.08	0.08	16	16	0.14	0.35	5	10
n	80	70	80	70	25	15	25	15
After heat stress								
$\bar{x}$	11.55[a]	13.76[a,b]	677[a]	281[a,b]	3.01[a]	2.69[a,b]	101[a]	103
SE	0.28	0.17	37	19	0.14	0.14	4	5
n	80	70	80	70	25	15	25	15

[a] significance ($p < 0.01$) between levels before and after heat stress.
[b] significance ($p < 0.01$) between surviving and non-surviving chickens.

cose, uric acid, triiodothyronine and thyroxine were significantly ($p < 0.01$) different before and after heat stress. In the surviving group, except for thyroxine, the changes were also significantly ($p < 0.01$) different following heat stress. While the differences in blood levels between the surviving and non-surviving chickens before heat stress were not significant, they were significantly ($p < 0.01$) different (except for thyroxine), following heat stress (tab. 2).

The blood levels of inorganic phosphate, total and ionized calcium, potassium and sodium in chickens before and after heat stress are shown in table 3. Except for ionized calcium and potassium in the surviving group, the analytes measured showed significant changes following heat stress. There were significant differences between surviving and non-surviving chickens in the serum levels of potassium before heat stress and in the serum levels of inorganic phosphate,

Tab. 3 Levels of electrolytes in the blood of broilers before and after heat stress.

	Analyte									
	Inorganic phosphate (mmol/l)		Total calcium (mmol/l)		Ca, ionized (mmol/l)		Potassium (mmol/l)		Sodium (mmol/l)	
	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors
Before heat stress										
$\bar{x}$	2.11	2.07	2.53	2.53	1.15	1.09	6.38	6.18[b]	143.6	143.3
SE	0.03	0.02	0.02	0.02	0.02	0.02	0.09	0.07	0.27	0.3
After heat stress										
$\bar{x}$	1.23[a]	1.60[a,b]	2.35[a]	2.45[a,b]	1.01[a]	1.10[b]	6.98[a]	6.12[a]	146.3[a]	146.9[a]
SE	0.04	0.05	0.02	0.02	0.01	0.01	0.07	0.07	0.5	0.4

n = 70–80
[a] significantly different ($p < 0.01$) between levels before and after heat stress.
[b] significantly different ($p < 0.01$) between surviving and non-surviving chickens.

Tab. 4 Total creatine kinase activity and the activities of creatine kinase isoenzymes, and their relative distribution in erythrocytes in surviving and non-surviving broilers before and during heat stress.

	Total creatine kinase (U/mg Hb)		Creatine kinase isoenzymes					
			BB (%)		MB (%)		MM (%)	
	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors
Before heat stress								
$\bar{x}$	25.1	30.6	2.91	2.35	15.1	12.9	82.0	85.2
SE	2.5	4.6	0.27	0.17	1.1	1.2	1.2	1.2
After heat stress								
$\bar{x}$	15.2[a]	37.3[b]	4.84[a]	2.66[b]	22.9[a]	14.2[b]	72.3[a]	83.1[b]
SE	2.2	4.7	0.57	0.17	2.0	3.0	2.3	3.0

n = 7–24
[a] significance levels ($p < 0.01$) between levels before and after heat stress.
[b] significance levels ($p < 0.01$) between surviving and non-surviving chickens.

Tab. 5 Rate of water consumption and haematocrit of survivors and non-surviving broilers before and during heat stress.

	Quantity					
	Water consumed (ml/h)		Haematocrit		Body temperature (°C)	
	Non-Survivors	Survivors	Non-Survivors	Survivors	Non-Survivors	Survivors
Before heat stress						
$\bar{x}$	24.3	26.0	0.315	0.303	40.8	40.7
SE	2.3	3.8	0.004	0.004	0.2	0.2
n	25	15	80	70	80	70
During/after heat stress						
$\bar{x}$	54.8[a]	71.7[a,b]	0.309	0.294	43.2	42.7
SE	6.8	13.4	0.004	0.004	0.4	0.4
n	25	15	80	70	80	70

[a] significance ($p < 0.01$) between levels prior to and after heat stress.
[b] significance ($p < 0.01$) between surviving and non-surviving chickens.

total and ionized calcium and potassium after heat stress (tab. 3).

The levels of total creatine kinase and its isoenzymes from the erythrocytes of surviving and non-surviving chickens before and after heat stress are shown in table 4. There were significant ($p < 0.01$) changes in the creatine kinase activity (total and isoenzymatic) following heat stress only in the non-surviving chickens. Although there were no significant differences between the surviving and the non-surviving before heat stress, there were significant differences in the erythrocyte levels of creatine kinase activity between these groups following heat stress (tab. 4).

The amount of water consumed, the blood haematocrit of surviving and non-surviving chickens and cloacal temperatures before and after heat stress are depicted in table 5. As seen and expected, heat stress caused the chickens to increase their water consumption, significantly more in the surviving group. There were no significant differences in the haematocrit between the groups either before or after heat stress (tab. 5).

The relative distribution of the two haemoglobin fractions before and after heat stress are shown in table 6 and figure 1. Following electrophoretic separation, 2 main peaks were seen: peak 1, in the region of human haemoglobin C and peak 2 in the region of human haemoglobin S. The relative distribution and consequently the ratios between the two fractions were significantly ($p < 0.01$) different between the surviving and the non-surviving chicken groups following heat stress. While the ratio between the two fractions changed slightly in the surviving group, it was significantly higher in the

Tab. 6 The relationship between haemoglobin composition and survivability of broilers subjected to heat stress.

	Haemoglobin (%)		Peak 1 / Peak 2
	Peak 1	Peak 2	
Surviving	55.5 ± 5.8	44.5 ± 4.8	1.25 ± 0.11
Non-Surviving	69.8 ± 7.1	30.2 ± 3.2	2.31 ± 0.28[a]

n = 9
[a] significance ($p < 0.01$) between levels before and after heat stress.

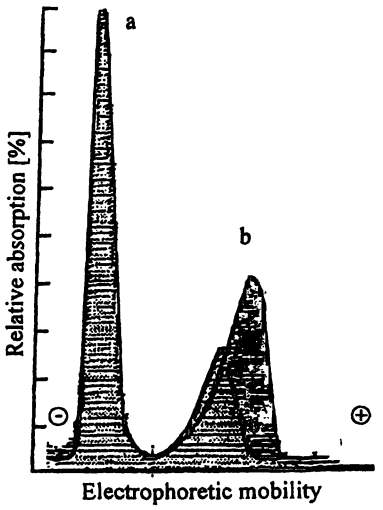

Fig. 1 Haemoglobin fractions separated by electrophoresis from surviving and non-surviving broilers following heat stress (relative distribution)
a: peak in the haemoglobin C region
b: peaks in the haemoglobin S region
▤ non-surviving ▢ surviving

non-surviving group, resulting from an increase of peak 1 and a decrease of peak 2, so that the mean ratio changed from 1.25 to 2.31.

The correlations between the various blood analytes are shown in table 7. Significant, high correlations (above 0.9000) were seen between uric acid and erythrocyte creatine kinase isoenzyme, and also between glucose and uric acid. Lower correlations were seen between glucose and ionized calcium ($r = 0.8427$), glucose and inorganic phosphate ($r = -0.8685$), triiodothyronine and glucose ($r = 0.7670$) and inorganic phosphate and total calcium ($r = 0.6851$).

Tab. 7 Correlation between blood analytes from broilers under heat stress.

Analytes	r ($\bar{x}$ ± SEM; n)
Glucose – uric acid	−0.9010 ± 0.089; 32
Glucose – ionized calcium	0.8427 ± 0.011; 24
Glucose – inorganic phosphate	−0.8685 ± 0.012; 32
Uric acid – creatine kinase BB	0.9460 ± 0.010; 18
Uric acid – creatine kinase MB	0.9560 ± 0.014; 18
Uric acid – creatine kinase MM	−0.9648 ± 0.013; 18
Inorganic phosphate – total calcium	0.6851 ± 0.080; 34
T_3 – glucose	0.7670 ± 0.091; 30

$p < 0.01$

Discussion

Heat stress and hyperthemia lead to panting and consequent respiratory alkalosis, with extreme metabolic changes resulting in death.

The objectives of the present study were to:

(1) better understand the sequence of events and metabolic problems leading to the death of the chickens caused by heat stress,

(2) search for biochemical quantities which correlate well with the ability of the chicken to survive heat stress and hopefully use them as markers for the evaluation of heat stress survivability.

With these objectives in mind a wide range of biochemical analytes representing the various aspects of metabolism, nutriton, renal, liver and cardiac functions were determined.

Table 1 shows the blood levels of various analytes that change in the same direction in both surviving and non-surviving groups. These analytes have no potential as markers of survivability, unless the changes vary greatly in their intensity between the two groups. On the other hand, analytes in which the changes occur in opposite directions, have a greater potential both in aiding the understanding of the mechanism leading to the death of the chicken, and as markers characterising the ability to survive heat stress.

Among the analytes showing such a pattern are glucose which was elevated in the surviving group and lowered in the non-surviving group. The lower glucose levels seen in the non-surviving chickens can be explained by possible reduced gluconeogenic activity, due to the accumulation of organic acids in response to the alkalosis. Alternatively, the observed decrease in gluconeogenesis may be due to its decrease in the kidney. The increased blood uric acid is evidence of an affect on the kidneys, indicating renal failure.

Another blood analyte showing a different pattern between surviving and non-surviving chickens (following heat stress), was uric acid, which decreased in the surviving and increased in the non-surviving groups. The significant increase of uric acid, the main end product of nitrogen metabolism in chickens (6, 15, 16), could indicate renal insufficiency in the non-surviving chicken, which could be secondary to heart function insufficiency (5, 15, 17).

The degree of renal insufficiency is not clear, since inorganic phosphate levels which normally increase in massive renal failure (5, 15, 17), decreased in this case for reasons to be discussed later. Potassium, which also increases in terminal renal failure (5, 15, 17), was increased in the non-surviving chicken. The reason for the small decrease in the blood level of uric acid in the surviving group could be either dilution of the blood because of the increased water uptake, or enhanced removal of uric acid from the blood due to faster blood circulation in the kidney.

The ratio of the two analytes – glucose and uric acid – can serve as a quantity for the evaluation of the degree of stress and consequently the sensitivity of the chickens to heat stress and their ability to survive it. While the ratios between the two analytes in both groups were

identical (34.5) before heat stress, they were much lower (18.3) in the non-surviving and higher (52.5) in the surviving chickens following heat stress. This ratio seems to be a more sensitive quantity, having a larger scale and being more accurate because of its dependence on two measurements.

Of interest is the quantity "creatine kinase activity" in the erythrocyte haemolysate. Since the role of this enzyme in the erythrocytes is not clear, it is possible that the activity seen is either of an enzyme related to the erythrocyte skeleton or to any other activity involved in the generation of adenosine triphosphate which will produce a signal of creatine kinase activity. The last possibility may fit with the observation that inorganic phosphate in the serum decreased during heat stress (tab. 3) and probably entered the erythrocytes (18).

In a previous study, we showed that total phosphates in the erythrocytes increased following heat stress (18). Furthermore, the role and importance of phosphorylated intermediates in the erythrocytes for the regulation of haemoglobin affinity to oxygen and carbon dioxide is well established. These intermediates are 2,3-bisphosphoglycerate in mammals and inositol phosphate in birds. The main extracellular buffer system consists of carbonates, while a major intracellular buffer consists of phosphates. While the regulation of the carbonates can be achieved by respiration and kidney excretion, the intracellular buffering strength can be adapted by mobilisation of phosphates into and out of the cells (5, 15, 18). It is therefore reasonable that phosphates entering the erythrocytes not only contribute to the buffering of the cytoplasm, but also to the regulation of the haemoglobin, which during panting has to deal more effectively with the high oxygen and low carbon dioxide levels; this latter regulation occurs by an increase in the level of phosphates in the red blood cells (5, 15, 18).

Thus, although it has not been proven, it is possible that some of these creatine kinase activities observed in the erythrocyte haemolysate originated from phosphorylated intermediates in the cells. Indeed, changes in the relative distribution of the various "isoenzymatic" peaks correlate with the survivability of chickens to heat stress.

The creatine kinase activity was further characterised. Heat treatment at 70 °C for 1 minute completely abolished this activity. Furthermore, dialysis of the haemolysate for 24 hours (exclusion pore size of 25 000) did not abolish the activity. The data suggest that this creatine kinase activity is associated with a large molecular mass, heat-sensitive molecule and that it is not dialysable, either because it is large, or because it is bound to another large molecule.

Measurement and characterisation of the haemoglobin revealed two fractions that are different from the normal haemoglobin fractions known in mammals. The avian haemoglobin fractions migrated in an electrophoretic field to regions where the pathological human haemoglobin S and C are found. Of interest was the observation that the relative distribution, and consequently, the ratio between the two haemoglobin peaks changed following heat stress, with a lower ratio in the surviving group and an elevated ratio in the non-surviving chickens. This phenomenon supports the theory that heat stress leads to changes of the haemoglobin, changes which are different between the surviving and non-surviving chickens exposed to heat stress.

The significance of the changes in the levels of creatine kinase activity and the haemoglobin fractions in relation to heat stress is not clear. Furthermore, it is not known whether any relationship exists between these changes. The fact that both changes are triggered by heat stress, and are highly correlated with survivability, suggests a relationship and a possible role in the mechanism of the response to and protection from heat stress.

Non-surviving chickens drank significantly less water than the surviving ones. It is of interest that despite the fact that water consumption was 2–3 times greater, it did not affect the haematocrit. This could be explained by the greater water loss by evaporation through the lungs, by the water loss in the faeces which became very watery during the heat stress, and by the very big water accumulation in the crop.

In conclusion, the present study shows some highly significant changes, which correlate highly with heat stress survivability. These traits relate to the ability to overcome the metabolic stress leading to renal insufficiency, and to maintain normal levels of glucose, ionized calcium and phosphorus. It seems that the surviving birds are more capable of coping the loss of carbon dioxide and high oxygen levels caused by hyperventilation, by manipulating the haemoglobin affinity for these gases; this may be related to the change in the haemoglobin subgroups and in those phosphate intermediates controlled or expressed by the creatine kinase activity in the erythrocytes.

This adaptability to acute hyperthermia could be a genetic characteristic. Therefore, those blood properties that are highly correlated with the ability to withstand heat stress may serve as genetic markers for the selection and development of a chicken more resistant to heat stress.

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