Involvement of Steroid Hormones, Corticosterone and Testosterone, in Synthesis of Heat Shock Proteins in Broiler Chickens

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Abstract: Large comb (LC) broiler cockerels, with high levels of testosterone and corticosterone, survived acute heat stress while small comb (SC) cockerels, with low levels of testosterone and corticosterone were more susceptible to heat stress and suffered higher mortality rates. This phenomenon was related to the greater ability of the LC and the lesser ability of the SC broilers to synthesize heat shock proteins (HSP), which are involved in acquisition and maintenance of thermotolerance. When broiler cockerels, selected for LC or SC, were exposed to acute heat stress, the synthesis of three HSP-hsp-90, hsp-70 and hsp-23 by peripheral blood leukocytes was elevated in both groups of broilers, but LC chickens responded with higher HSP synthesis than did SC chickens. One of the normal cellular proteins, actin, was depressed during the heat stress over 105 minutes. To determine whether steroid hormones, testosterone and corticosterone, influence the expression of HSP in chickens, exogenous testosterone and corticosterone were implanted in LC capons and metryrapone-fed LC capons, respectively. The plasma testosterone was raised in the testosterone-treated capons, was depressed in the capons and metryrapone-fed capons and was indicated indirectly by growth rate of the comb. The heat-induced synthesis of HSP was depressed by caponization and depressed further in the capons fed metryrapone, which blocks the synthesis pathway of endogenous corticosterone in adrenal glands. The exogenous testosterone and to a lesser degree, corticosterone, stimulated the expression of HSP in heat-stressed capons. The results suggested that the steroid hormones, testosterone and corticosterone, are involved in the expression of HSP, which are associated with acquired and maintained thermotolerance in domestic chickens.

Key words: Broiler cockerels, capon, corticosterone, testosterone, heat shock proteins, leukocytes, heat stress, steroid hormones

Introduction
The heat shock phenomenon is the manifestation of a response to exposure to elevated ambient temperature, which will induce or enhance gene expression of a small group of polypeptides referred to as heat shock proteins (HSP) and this response has been recognized in almost every living organism (Ashburner and Bonner, 1979; Craig, 1985; Lindquist, 1986). Many researchers have been able to show evidence that HSP play very important roles in cellular homeostasis (Lindquist and Craig, 1988). For example, any environmental insult, such as hyperthermia, will induce cells to die as a result of accumulation of abnormal or damaged proteins (Magun and Fennie, 1981; Nguyen et al., 1989) and denaturation of enzymes, including DNA polymerase poly (ADP-ribose) synthetase, Na⁺-K⁺-ATPase, Ca²⁺-ATPase, NADPH oxidase and β-galactosidase (Bensaude et al., 1990). To survive a variety of stressors, cells or organisms have to deal with protein dysfunction by degrading and/or reforming the proteins. The abnormal proteins are usually degraded by the ATP-dependent ubiquitin pathway (Hershko, 1983), which acts as a protease (Fried et al., 1987; Rechsteiner, 1987). Ubiquitin, however, is also a heat shock protein (Bond and Schlesinger, 1985). It was proposed that HSP, encoded by HSP genes, may protect the cells through the prevention of protein degradation (Minton et al., 1982), through the rapid elimination of abnormal proteins and aggregates (Pelham, 1986), or through reconstitution of the denatured proteins (Gaitanaris et al., 1990) possibly via refolding (Gaitanaris et al., 1990) or via intramolecular interactions (Zhu et al., 1989) by ATP-dependent hydrolysis (Ostermann et al., 1989). Therefore, the HSP have been well characterized as being involved in acquisition and maintenance of thermotolerance (Lindquist, 1986). The physiological response to heat stress of animals such as chickens has been investigated extensively (Edens and Siegel, 1975; Edens, 1978; McCormick et al., 1979, 1980; Siegel and Gould, 1982). Edens and Siegel (1975) demonstrated that cardiovascular collapse, which is the ultimate cause of heat stress mortality in chickens (Whittow et al., 1964), was preceded by acute adrenal cortical insufficiency characterized by depletion of corticosterone and cessation of its production (Edens, 1978). However, the cellular and molecular mechanism(s) involved in the chicken's failure to survive heat stressors remain largely unresolved.
Studies of heat shock responses have clearly indicated that a 90 kDa heat shock protein (hsp-90) is associated with steroid receptors, including estrogen/estradiol, progesterone, androgens, glucocorticoids and mineralocorticoid receptors. Hsp-90 represses the receptor function by capping the receptor DNA-binding site (Baulieu, 1987). Binding of cognate hormone results in a transformation/activation of unactivated/untransformed receptors (9 S) into activated receptors (4 S) by releasing hsp-90 (Jolab et al., 1984). The 4 S active receptor can then bind to DNA and trigger the hormonal responses. Research has shown that untransformed receptor complex contains hsp-70 in addition to hsp-90 in progesterone receptors of birds (Kost et al., 1989) and humans (Estes et al., 1987; Sanchez et al., 1990). A 59 kDa protein was also found to associate with progesterin, androgen and glucocorticoid receptors with undefined function (Tai et al., 1986; Renoir et al., 1990; Sanchez et al., 1990).

It has been demonstrated that several HSP can be induced by administration of steroids and their analogues in animal tissue or cells, including fruit flies (Beaulieu et al., 1989), mouse (Ramachandran et al., 1988; Shyamala et al., 1989) and chicken (Baez et al., 1987). Steroid hormones appear to be associated with the gene regulation of HSP synthesis, but there is little information available on the physiological relevance of the steroid influence on HSP synthesis. This study was conducted to examine the effect of a sex hormone-testosterone and the avian stress hormone-corticosterone on the expression of HSP in broiler chickens during acute heat stress.

**Materials and Methods**

**Animals:** In both experiments, commercial Arbor Acres X Arbor Acres broiler chickens were hatched at the North Carolina Agriculture Research Service poultry hatchery. Gender of chicks was determined by wing feather development of the hatchlings. Males were identified at hatching by short primary wing feathers while females had long primary feathers. Males only were selected for these experiments. These experiments were conducted under the supervision of the North Carolina State University Institutional Animal Care and Use Committee.

In Experiment 1, day-old chicks were placed into a thermoneutral environment in a battery brooder with water and feed available on an *ad libitum* basis. At 3 weeks of age, the broiler cockerels were phenotypically selected for large comb (LC) and small comb (SC). As an added measure, blood plasma was collected for determination of both testosterone and corticosterone in non-stressed broilers. The selected chickens were exposed to acute heat stress *in vivo* at 41°C for 105 minutes. The colonic temperature (*Tb*) was measured by a tele-thermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH).

In Experiment 2, 2-wk old LC broiler cockerels were selected and were cauponized by a procedure similar to that described previously. The broiler chickens were anesthetized with an intramuscular injection of a mixture of Ketamine (20 mg/kg) and Xylazine (10 mg/kg) and when they were in deep anesthesia, they were cauponized. Briefly, bilateral incisions were made through iodine-alcohol sanitized skin anterior to the last vertebral rib, a mechanical retractor was inserted to spread the ribs 1.0 cm, the caudal thoracic air sac was perforated and with the extraction apparatus, testes were isolated on each side of the bird and then removed from the 24-hour-fasted males. The controls were sham-operated undergoing the same procedure without extraction of the testes. The rib incision was closed with a single sterile suture and skin was closed with two stainless steel wound clips. The capons were then allowed to recover from the surgery for one week and were given two injections of gentamicin (1 mg/kg IM) to limit inflammation and bacterial infection after cauponization. The capons were assigned randomly to different treatments and intact LC cockerels were used as positive controls. One group of intact SC cockerels was also included in this experiment for use in comparison of comb growth of the LC intact, LC cauponized and LC cauponized steroid/metyparone-treated groups. There were five groups of seven chicks, the LC control, the capons, the capons implanted with a testosterone propionate pellet (5 mg/pellet for 21-day release; Innovative Research of America, Toledo, OH), the capons fed metyparone of 500 mg per kg feed (Sigma Chemical Co., St. Louis, MO) and the metyparone-fed capons implanted with a corticosterone pellet (5 mg/pellet for 21-day release; Innovative Research of America, Toledo, OH). There were seven selected SC cockerels which served as a sixth group as a negative control group, which did not have implanted steroid-delivering pellets. The concentration of feed-delivered metyparone was reported to be the optimal dosage for a wide range of body weights (0.35-5.2 kg) of chicks to block the endogenous corticosterone synthesis pathway in adrenal glands (Dominguez and Samuels, 1963; Gross, 1990). At the end of the first, second and third week after implantation, the LC birds were subjected to acute heat exposure at 41°C for 60 minutes in a heat-chamber. Blood samples were taken for analysis of leukocyte HSP expression. At 1 week after the depletion of hormones from the pellets, the LC chickens were heat stressed at 41°C for 60 minutes. Comb size of all chickens, including the SC negative controls, was recorded at two and three weeks after pellet implantation into the LC cockerels.

**Heat shock, isolation and radio-labeling of peripheral blood leukocytes:** The procedures used in Experiment 1 and 2 were described elsewhere (Wang and Edens, 1993a,b and 1998). In brief, blood samples were taken from the heat-stressed chickens for *in vivo* heat shock response and from the same treatment of chickens at
non-heat-stress condition for in vitro heat shock response. After isolation of peripheral leukocytes with Histopaque (Sigma Chemical Co., St. Louis, MO), the cells were suspended and labeled in eagle's methionine-free essential medium (MEM-Met) (Lineberger Cancer Center, University of North Carolina, School of Medicine, Chapel Hill, NC) containing 100μCi/ml of L-[35S] methionine (1085 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA) at 37°C for 30 minutes. The isolated cells for in vitro heat stress were incubated at 41°C for 60 minutes before labeling. The labeling was then terminated by adding cold (4°C) Hank’s balanced salt solution (HBSS) lacking Ca2+ and Mg2+ (Sigma Chemical Co., St. Louis, MO). The cells were pelleted by centrifugation. After three washings with HBSS the cells were lysed in the sample lysis buffer (Laemmli, 1970) with addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). The cellular proteins were heat-denatured at 95°C for 5 minutes and aliquots were stored at -70°C. The radioactivity of 35S-methionine-labeled proteins was determined by trichloroacetic acid (TCA) precipitation method (Wang and Edens, 1993ab and 1996). In short, equal amount of aliquots (5 μl) from each sample was precipitated on filter paper by 10% TCA. After three washings to remove the free 35S with ethanol, the radioactivity of 35S-methionine-labeled cellular proteins, precipitated on the filter paper, was counted with a Beckman LS-100 Liquid Scintillation Counter (Beckman Instruments, Inc., Irvine, CA).

Polycrylamide gel electrophoresis and autoradiography: The cellular proteins were separated in one-dimensional sodium doceyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) based on the procedure described by Laemmli (1970). Equal amounts of proteins were loaded onto the gel with equal amounts of radioactivity (cpm). Prestained protein markers with known molecular weights were applied to calculate the size of radio-labeled proteins. The protein markers included lysozyme (14 kDa), trypsin inhibitor (20.4 kDa), carbonic anhydrase (29 kDa), alcohol dehydrogenase (39 kDa), bovine serum albumin (68 kDa) and phosphorylase B (97 kDa) (Integrated Separation System, Hyde Park, MA). The gel was dried on gel filter paper with a Bio-Rad model 583 gel dryer (Bio-Rad Laboratories, Melville, NY) and exposed to Kodak X-OEM AR film for autoradiography. The relative concentration of the radio-labeled cellular proteins was quantified with a 300 A Scanning Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

Plasma testosterone and corticosterone analysis: Plasma testosterone and corticosterone were determined with 125I-labeled commercial kits obtained from DPC (Diagnostic Products Corp., Los Angeles, CA) and Ventrex Laboratories (Ventrex Laboratories, Inc., Portland, ME), respectively, by following the manufacturers’ procedures.

Statistical analysis: Data analysis was performed with analysis of variance (ANOVA) using the general linear model (GLM) procedure of the statistical analysis system (SAS, 2001). Differences between or among means were determined using probabilities generated by least square means.

Results

Experiment 1: Comb-size-related difference in synthesis of HSP in peripheral blood leukocytes from large comb (LC) and small comb (SC) heat-stressed broilers: Phenotype selected LC and SC broiler chickens were further segregated on the basis of blood plasma testosterone and corticosterone (Table 1). The LC broilers tended to have significantly greater levels of plasma testosterone and corticosterone than did the SC broilers. To determine whether the lower mortality rate of phenotype-selected LC cockerels was associated with their capability to synthesize HSP, the LC and SC cockerels were heat challenged at 41°C for 105 minutes. The HSP and cellular protein responses of the peripheral leukocytes isolated from one pair of the heat-stressed LC and SC cockerels are shown in Fig. 1 as an example of the divergent responsiveness of the chickens with LC and SC comb phenotypes. Three major HSP with molecular weights of 90 kDa, 70 kDa and 23 kDa were induced in a time-dependent manner in both LC and SC cockerels. Summarized in Fig. 2 are the mean densitometric values of HSP synthesis in both LC and SC cockerels. The synthesis rate of different HSP increased in response to the elevated ambient temperature and elevated Tb in both LC and SC broilers regardless of comb phenotype. The average levels of the major HSP, hsp-90, hsp-70 and hsp-23 (Fig. 2A, 2B and 2C, respectively) were significantly greater (P ≤ 0.05) by a factor of about 25% in heat-stressed LC than in heat-stressed SC cockerels at 60 and 105 minutes of exposure. Increased synthesis of hsp-90, hsp-70 and hsp-23 was related to increased Tb in both LC and SC cockerels (Fig. 2E). The synthesis of the 45 kDa actin, one of the major normal cellular proteins, was depressed significantly by heat stress at 105 minutes of exposure in the LC cockerels (Fig. 2D), but there was little difference between LC and SC for the actin depression before 105 minutes heat stress exposure. In both LC and SC broilers, there was a time-dependent increase in Tb, which increased in a parallel pattern between the pairs of LC and SC chickens (Fig. 2E). Yet, Tb may not account for the difference in HSP levels between the LC and SC cockerels since the average body temperatures between LC and SC cockerels were not significantly different. However, Tb of the LC birds did tend to be slightly greater than those of the SC cockerels at 60 and 105 minutes exposure times to heat stress (Fig. 2E), but the relatively higher Tb in LC cockerels might have reflected the fact that four of the seven SC
birds had died between 60 and 105 minutes exposure to the elevated ambient temperature. Therefore SC survivors might have lived longer because they had fewer than lethal TB at 106 minutes of exposure.

Experiment 2: Influence of testosterone and corticosterone on synthesis of HSP in peripheral blood leukocytes of large comb intact and caponized broiler cockerels: After the first week of treatment, hsp-80 and hsp-70 were increased slightly in all chickens in response to the first exposure to acute heat stress, but hsp-23 was not induced (data not shown). The hsp-80 and hsp-70 responses (Fig. 3A and 4A) after the second week of treatment were similar but of a greater magnitude greater than that of the first week of treatment and hsp-23 (Fig. 5A and 5B) was induced significantly in heat stressed capons, testosterone- and corticosterone-implanted capons. There was a significant time effect in which there was an augmented induction of hsp-70 and hsp-60 and showed that repeated heat exposure from one to three weeks after implantation of steroid pellets, the greatest induction of the HSP was at three weeks (Fig. 3A, 3B, 4A and 4B). Hsp-23 synthesis also appeared to be influenced more by the presence of testosterone in the LC cockerels than by the presence of corticosterone. In this investigation, exposure to high temperature stress for only 60 minutes was not sufficient to cause a loss of actin (Fig. 6) as seen in Experiment 1 after 105 minutes at 41°C. Densestometric analysis indicated that capons, treated with testosterone, synthesized significantly larger quantities of HSP while the metyrapone-fed capons synthesized smaller quantities of HSP when compared to the intact control. Corticosterone, however, stimulated significantly the synthesis of HSP back to the unstressed control level in the metyrapone-fed capon. Under the acute heat stress environment, the non-treated capons synthesized significantly smaller quantities of hsp-80, hsp-70 and hsp-23 than did the control. Testosterone implantation stimulated the synthesis of the HSP resulting in the greatest quantities among the treatments. Metyrapone-feeding to capons, on the other hand, depressed the expression of the HSP to the lowest level compared to the other chickens. This response to metyrapone could be due to the lack of available testosterone and corticosterone in circulation, since administration of corticosterone to the metyrapone-fed capons stimulated the synthesis of HSP back to the control levels. These experimental treatments had little effect on the control levels of HSP in non-stressed birds. One week after depletion of the steroids from the pellets, the testosterone and corticosterone-treated chickens failed to show a continued effect on the synthesis of HSP even under the heat stress environment (Fig. 3C, 4C and 5C). Growth of the intact and caponized LC cockerel combs and intact SC cockerel combs were measured as a parameter to indicate indirectly plasma concentration of testosterone (Fig. 6A and 6B). After hormone treatments for 2 and 3 weeks, caponization significantly (P < 0.05) depressed the comb growth rate of LC cockerels to a level similar to that of SC birds. Testosterone implantation stimulated comb growth in the LC capons to a level above the intact LC and SC controls. The growth rate was not affected in metyrapone and corticosterone-treated capons when compared to the capons alone (Fig. 3a and 3b).
Plasma corticosterone (Table 2) and testosterone (Table 3) were measured in the intact LC and SC cockerels at 1, 2 and 3 weeks after steroid implantation in the LC capons and again at one week past expected exhaustion of hormone pellets in the LC capons. Similar to the results for corticosterone and testosterone in the intact LC and SC cockerels in Experiment 1, the intact LC cockerels in Experiment 2 had significantly greater plasma concentrations of corticosterone and testosterone than did SC cockerels (Tables 2 and 3). Caponized LC cockerels with and without hormone implantation showed a wide range of plasma corticosterone levels. Corticosterone levels declined in caponized LC cockerels to a level similar to intact SC cockerels. Testosterone implantation caused a rebound in plasma corticosterone levels almost to the level of the intact LC cockerels. Metyrapone feeding caused plasma corticosterone levels to decline significantly to a level that was just barely detectable with the radioimmunoassay utilized in this investigation and implantation of the corticosterone pellet into the metyrapone fed LC cockerels elevated plasma corticosterone back to a level slightly greater than that found in intact LC cockerels. However, exhaustion of the corticosterone in the pellet with continued feeding of metyrapone allowed plasma corticosterone to decline to the level of the metyrapone only group (Table 2). In the intact LC cockerels, plasma testosterone increased 2 fold (228.479 pg/ml) from 4-7 weeks of age (Table 3). Caponization caused plasma testosterone levels in LC cockerels to decrease to non-detectable levels within one week post-caponization (Table 3), but implantation of a testosterone pellet into LC capons caused plasma testosterone levels to increase about 1.5 fold the level of testosterone in intact LC cockerels (Table 3). Exhaustion of the testosterone in the implanted pellet in LC capons.
Fig. 3: Densitometric analysis of heat shock protein 90 (hsp 90) in response to in vivo heat shock of peripheral blood leukocytes in large comb control (LC, cont) intact broiler cockerels, LC capons (Cap) and LC Cap given subcutaneous pellets of Testosterone (Test), Metapyrone (Metry), an enzyme inhibitor that blocks endogenous synthesis of corticosterone [Cort]), or Metyr plus Cort at one week post-caponization (three weeks of age) with acute exposure for 60 minutes to high ambient temperature (41°C) at 2 (Fig. 3A) and 3 weeks (Fig. 3B) post pellet implantation and at one week after pellet steroid exhaustion (Fig. 3C) at 4 weeks post-pellet implantation. Unlike lower case letters above the histogram bars indicate significant differences (P ≤ 0.05) among treatment means (n = 7 per treatment).
Fig. 4: Densitometric analysis of heat shock protein 70 (hsp 70) in response to in vivo heat shock of peripheral blood leukocytes in large comb control (LC, cont) intact broiler cockerels, LC capons (Cap) and LC Cap given subcutaneous pellets of Testosterone (Test), Metyrapone (Metyr), an enzyme inhibitor that blocks endogenous synthesis of corticosterone [Cort]], or Metyr plus Cort at one week post-caponization (three weeks of age) with acute exposure for 60 minutes to high ambient temperature (41°C) at 2 (Fig. 4A) and 3 weeks (Fig. 4B) post pellet implantation and at one week after pellet steroid exhaustion (Fig. 4C) at 4 weeks post-pellet implantation. Unlike lower case letters above the histogram bars indicate significant differences (P ≤ 0.05) among treatment means (n = 7 per treatment).
Fig. 5: Densitometric analysis of heat shock protein 23 (hsp 23) in response to in vivo heat shock of peripheral blood leukocytes in large comb control (LC, cont) intact broiler cockerels, LC capons (Cap) and LC Cap given subcutaneous pellets of Testosterone (Test), Metyrapone (Metyr; an enzyme inhibitor that blocks endogenous synthesis of corticosterone [Cort]), or Metyr plus Cort at one week post-caponization (three weeks of age) with acute exposure for 60 minutes to high ambient temperature (41°C) at 2 (Fig. 5A) and 3 weeks (Fig. 5B) post pellet implantation and at one week after pellet steroid exhaustion (Fig. 5C) at 4 weeks post-pellet implantation. Unlike lower case letters above the histogram bars indicate significant differences (P ≤ 0.05) among treatment means (n = 7 per treatment).
Fig. 6: Densitometric analysis of actin in response to \textit{in vivo} heat shock of peripheral blood leukocytes in large comb control (LC; cont) intact broiler cockerels, LC capons (Cap) and LC Cap given subcutaneous pellets of Testosterone (Test), Metyrapone (Metyr; an enzyme inhibitor that blocks endogenous synthesis of corticosterone [Cort]), or Metyr plus Cort at one week post-caponization (three weeks of age) with acute exposure for 60 minutes to high ambient temperature (41°C) at 2 (Fig. 6A) and 3 weeks (Fig. 6B) post pellet implantation and at one week after pellet steroid exhaustion (Fig. 6C) at 4 weeks post-pellet implantation. There were no significant differences among treatments within a time for leukocyte actin levels.
observed that LC broiler cockerels were more heat resistant than SC cockerels (Davis et al., 1991). A similar observation was made when LC and SC birds were segregated phenotypically on the basis of high plasma testosterone (HT) and low plasma testosterone (LT) (Edens and Wang, 1990; Wang and Edens, 1993b). The HT was associated with higher concentrations of plasma corticosterone and created a condition in which the LC cockerels were more resistant to the detrimental effects of heat stress. The mechanism(s) of improved heat resistance in the phenotype-selected LC birds, with HT and high corticosterone levels, was not known. During heat stress, cardiovascular failure in chickens (Whittow, 1964) results from acute adrenal cortical insufficiency (Edens and Siegel, 1975; Edens, 1978), which appears to precipitate early heat-related death. Based on the finding that synthesis of HSP may be related to development of thermotolerance, it was hypothesized that the phenotype-related heat resistance may be associated with the cockerel's ability to synthesize HSP.

In this investigation, it was found that peripheral blood leukocytes, isolated from the phenotype-selected LC cockerels synthesized more HSP than leukocytes from SC cockerels when they were exposed to acute heat stress in vivo (Fig. 1 and 2). Enhanced expression of HSP is a temperature-dependent trait in birds (Wang and Edens, 1993a, b and 1998) and body temperature might contribute to greater quantities of HSP synthesized in the LC bird. However, there was little difference between Tb of the LC and SC cockerels (see Fig. 2E). Therefore, other in vivo factors, such as the plasma corticosterone and testosterone, in addition to hyperthermia, appear to be involved in the gene expression of HSP under the heat stress environment. Based on the results from this investigation, plasma corticosterone and testosterone do participate in the regulation of HSP synthesis. The pattern of HSP expression was altered in Experiment 2 by the different treatments, caponization, metyrapone-feeding and implantation of testosterone and corticosterone. Metyrapone acts as an inhibitor of corticosterone synthesis in adrenal glands by inhibiting 11 beta-hydroxylase activity (Domínguez and Samuels, 1963; Gross, 1990). The concentration of metyrapone used in this study was found to be the optimal dosage for chickens with body weights ranging from 0.35-5.2 kg.

**Table 1:** Plasma testosterone and corticosterone in phenotype selected large comb (LC) and small comb (SC) broiler chickens

<table>
<thead>
<tr>
<th>Comb Size</th>
<th>Testosterone, pg/ml</th>
<th>Corticosterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Comb</td>
<td>257±19 <em>a</em></td>
<td>2.9±0.2 <em>b</em></td>
</tr>
<tr>
<td>Small Comb</td>
<td>108±9 <em>b</em></td>
<td>1.3±0.2 <em>b</em></td>
</tr>
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*a*In a column, means with unlike lower case superscripts differ significantly (P<0.05).

resulted in a decrease in plasma testosterone to an undetectable level within one week of exhaustion of the pellet.

**Discussion**

This study examined the interaction of steroid hormones, specifically, testosterone and corticosterone and their effect on HSP synthesis, comb growth and heat resistance in phenotype-selected large comb (LC) and small comb (SC) broiler cockerels. In an earlier study, in a simulated field trial with 4500 cockerels, it was

**Fig. 7:** A comparison of comb growth of large comb (LC) and small comb (SC) intact broiler cockerels and LC capons (Cap) given subcutaneous pellets of testosterone (Test), metyrapone (Metyr; an enzyme inhibitor that blocks endogenous synthesis of corticosterone [Cort]) or metyrapone plus corticosterone at one week post-caponization (three weeks of age) with comb measurements at two and three weeks after implantation of the steroid hormone pellets. Bars on the histogram represent mean (±SEM) of comb length (cm) and height (cm) at five and six weeks of age. Unlike lower case letters above the histogram bars indicate significant differences (P ≤ 0.05) among treatment means (n = 7 per treatment).
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Table 2: Influence of caponization and hormone supplements via pellet implants on plasma levels of corticosterone (ng/ml) in large comb (LC) phenotype (positive control) and the small comb (SC) phenotype (negative control) at 1, 2, and 3 weeks after pellet implantation in LC birds and at 1 week after exhaustion of hormone in the implants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Implant Exhaused</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LC</td>
<td>SC</td>
<td>LC</td>
<td>SC</td>
</tr>
<tr>
<td>Control</td>
<td>2.3^a</td>
<td>1.5^a</td>
<td>2.4^a</td>
<td>1.6^a</td>
</tr>
<tr>
<td>Capan</td>
<td>1.5</td>
<td>1.4</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Capan+Testosterone</td>
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<td>2.2</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Capan+Metyrapone</td>
<td>0.8</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Capan+Metyrapone+Corticosterone</td>
<td>4.3</td>
<td>4.1</td>
<td>4.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

^a,b,c In a column, means with unlike lower case superscripts differ significantly (P = 0.05). ^A,B In a row, means with unlike upper case superscripts differ significantly (P = 0.05).

Table 3: Influence of caponization and hormone supplements via pellet implants on plasma levels of testosterone (pg/ml) in large comb (LC) phenotype (positive control) and the small comb (SC) phenotype (negative control) at 1, 2, and 3 weeks after pellet implantation in LC birds and at 1 week after exhaustion of hormone in the implants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Implant Exhausted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC</td>
<td>SC</td>
<td>LC</td>
<td>SC</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>Capan+Metyrapone+Corticosterone</td>
<td>&lt;10^a</td>
<td>&lt;10^a</td>
<td>&lt;10^a</td>
<td>&lt;10^a</td>
</tr>
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^a,b,c In a column, means with unlike lower case superscripts differ significantly (P = 0.05). ^A,B In a row, means with unlike upper case superscripts differ significantly (P = 0.05).

(Gross, 1990). Metyrapone-feeding to the capons consistently depressed the synthesis of three HSP- hsp-90, hsp-70 and hsp-23 to the lowest levels among the treatments in all of the weekly measurements. When the metyrapone-fed caponized cockerels were given exogenous corticosterone in a silastic pellet, the HSP synthesis rate returned to the non-stressed control level. This demonstrated the involvement of plasma corticosterone in expression of HSP in heat-stressed chickens. The LC capons showed low synthesis rates of the HSP compared to the control, but the rate was still higher than in the metyrapone-fed capons at the third week of treatment (Fig. 3, 4 and 5).

The testosterone-treated capons synthesized the greatest quantities of HSP among all of the treatments during 3 weeks of treatment indicating that testosterone played a major role in the induction of HSP in males. The plasma concentration of testosterone followed a pattern previously associated with LC/HT and SC/LT (Wang and Edens, 1993b). Thus, the comb size can be used as an indicator for testosterone levels in circulation (Tables 1 and 3). The growth rate of comb was severely depressed by caponization, but testosterone administration significantly stimulated the comb growth in the LC capons. On the other hand, the testosterone-stimulated HSP synthesis may also be due to the partial involvement of plasma corticosterone because the concentration of plasma corticosterone was correlated with the plasma testosterone in this study and in earlier studies (Edens and Wang, 1990; Wang and Edens, 1993b). However, the steroid hormone treatments in the non-heat-stressed LC capons showed little effect on the basal levels of HSP (hsp-90, hsp-70 and hsp-23) in the leukocytes, which is similar to observations reported by Fisher et al. (1986), who found that treatments of testosterone and dexamethasone did not stimulate HSP synthesis in Chinese hamster ovary cells under non-heat-stress condition.

In male rats, castration resulted in the inhibition of hsp-70 accumulation in the heart of strenuously exercised animals, but intact and dihydrotestosterone-treated orchidectomized animals showed a significant increase in cardiac hsp-70 mRNA and protein (Milne et al., 2006). Paroo et al. (2002) found that when strenuous exercise is used to elevate cardiac hsp-70, male rats were more resistant to ischemia-reperfusion and lethal stress. This is an important consideration as the heart of male broiler chickens, compared to female broilers, because males appear to be more susceptible to heart failure during heat stress (Whittow et al., 1984, Edens, 1978; Edens and Siegel, 1975). During heat stress, broiler chicken heart rates can increase from a resting rate of about 180-220 beats per minute to exceed a rate of more than 350-450 beats per minute along with respiration rates increasing to more than 300 breaths per minute and sustain those rates until respiratory exhaustion and heart failure occur when body temperature exceeds 45.5°C. Heat stress can stimulate the chicken heart comparable to that experienced by the heart of male rats subjected to strenuous exercise. In the current investigation, testosterone, the male sex steroid hormone, appeared to play a major role in the
expression of hsp-70 in peripheral blood leukocytes and presumably in other tissues also.
Edens and Siegel (1975) demonstrated that acute adrenal cortical insufficiency also contributed to heat related mortality in young chickens. Thus, the contribution of corticosterone to the induction and accumulation of hsp-70 and other HSP is a question that remains to be answered. Corticosterone and testosterone treatments in this study affected gene expression of HSP only during heat shock suggesting another role of these steroid hormones during periods of exposure of the animal to severe conditions such as heat stress.
It has been documented that steroid receptors, including glucocorticoid receptor, contain hsp-70 (Estes et al., 1987; Kost et al., 1989; Sanchez et al., 1990; Perdew and Whitelaw, 1991) as well as a few other small proteins (23-63 kDa; Tai et al., 1986; Renoir et al., 1990; Sanchez et al., 1990) in addition to hsp-90 protein. HSP may be released from the receptors by steroid hormones binding to the receptors (Baulieu, 1987; Pratt et al., 1989). It is possible that the synthesis of hsp-90, hsp-70 and hsp-23 in response to heat stress by manipulation of plasma corticosterone and/or testosterone, noted in the present study, could be regulated through the binding of the steroid hormones to receptors in peripheral leukocytes. Glucocorticoid receptors have been identified in the peripheral blood leukocytes (Lippman and Barr, 1973; Junker, 1983; Martins et al., 1987), but the presence of testosterone receptor in avian leukocytes has not been reported. However, the involvement of testosterone in HSP induction, at least in the peripheral blood leukocytes in this investigation, might not be associated with the classical cytoplasmic androgen receptor but might be associated with a plasma membrane testosterone receptor in peripheral blood leukocytes (Benten et al., 1999).
In human and murine lymphocyte cell lines treated with dexamethasone, synthesis of hsp-90 was stimulated at the transcriptional level (Eiser et al., 1988) as well as at the translational level (Norton and Latchman, 1989). It was also noted that the expression of hsp90 was receptor-dependent in the lymphocytes (Eiser et al., 1988; Norton and Latchman, 1989). In addition, sex steroids have been shown to stimulate the synthesis of hsp-80 in mouse tissues (Ramachandran et al., 1988; Shyamala et al., 1989) and hsp 106 in chicken (Baez et al., 1987). Additionally, a number of HSP, especially the small molecular weight of 23-28 kDa proteins, have been induced in fruit flies by steroid hormone stimulation (Ireland and Berger, 1982; Ireland et al., 1982; Vitek and Berger, 1984) as well as by heat shock (Vitek and Berger, 1984).
In this study, it was shown that heat resistance in LC cockerels was associated with the plasma concentrations of corticosterone and testosterone, which appeared to regulate the synthesis of HSP. The question remains as to how HSP are involved in the lower mortality rate, i.e. higher thermoresistance in the heat-stressed LC birds. It has been suggested that heat stress causes cardiovascular collapse due to depletion of adrenal storage (Edens and Siegel, 1975), leading to the animal’s death (Whittow et al., 1964). However, the mechanism involved is far from clear on either a cellular or a molecular basis. Studies of heat shock response have revealed that the collapse of the intermediate filament cytoskeleton in heat shocked Drasophila cells resulted in large aggregates in the cell nucleus (Falkner et al., 1981; Walter and Biessemann, 1984; Walter et al., 1990). In this study and previous works from this laboratory, we have shown that with severe heat stress, actin and other structural proteins in cells begin to degrade and this is correlated with rapid increases in HSP. It has been noted that the heat shock-induced cell protein collapse and aggregates occurred because HSP were not induced (Walter et al., 1990), but with induction of HSP, such as hsp-70, damaged protein can be repaired. These observations imply that synthesis of HSP is related to the cell’s ability to survive. The failure of HSP induction could be destructive to the cells since induction of HSP appears to be essential to prevent the intermediate filament collapse in mammalian cells (Welch and Mizzen, 1988; Shyy et al., 1989). It has been assumed that this same mechanism occurs in chicken cells that are unable to synthetize sufficient quantities of HSP. Thus, testosterone induction of greater levels of HSP, possibly, is responsible for improved heat tolerance in LC males.
Stressful insults, such as heat, allow formation of damaged proteins (Magun and Fennie, 1981) and denatured enzymes, such as DNA polymerase, NADPH oxidase and Na⁺-K⁺-ATPase (reviewed in Bensaude et al., 1990), which lead to cell death. These denatured proteins and enzymes must be removed in order to normalize cellular metabolism and function. Evidence has been reported that HSP protect the cell by preventing the accumulation of denatured proteins (Minton et al., 1982) and eliminating the abnormal proteins and aggregates (Pelham, 1986). The dysfunctional proteins may even be reconstituted by the HSP (Gaitanaris et al., 1990).
The heat resistance associated with steroid hormones, shown in this study, appear to be associated with induced HSP, since the development of thermotolerance is very closely related with the induction of HSP (Landry et al., 1982; Subjeck et al., 1982; Tomaso etc., 1983; Li and Laszlo, 1985; Lindquist, 1988). When exposed to a severe heat environment at 45°C or above, cells were unable to survive with hsp-70 antibodies microinjected into cultured fibroblasts (Riabowski et al., 1988) and neurons (Khan and Sotelo, 1989). Similarly, mouse oocytes microinjected with hsp-70 mRNA survived better.
during heat challenge than those given hsp-70 antisense mRNA as controls (Hendrey and Kola, 1991). These results demonstrated that HSP are necessary for cells to increase their resistance to heat.

In summary, the higher resistance to heat stress noted in the phenotype-selected LC broiler cockerels is associated with higher plasma concentrations of steroid hormones, corticosterone and testosterone, than found in SC cockerels. The increased thermotolerance may be gained via the synthesis of cellular proteins encoded by heat shock genes in the peripheral blood leukocytes stimulated by steroids during heat stress. The HSP perform the function of thermostolerance development through the prevention, elimination, or reconstitution of denatured proteins and enzymes accumulated during heat shock exposure.

References


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