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13. ABSTRACT *(Maximum 200)*

These experiments determined if estrogen (E_2) supplementation in (a) menstruating young women during the follicular phase of their menstrual cycle, or (b) ovariectomized female rats would promote heat loss in women and enhance thermotolerance in animals. Women [11 control (P), 10 experimental (e)] performed cycle exercise at 60% VO_2 max in a cool (25°C) room for 20 min. Neither sweating threshold (36.97±0.15°C in P vs 36.9±0.22°C in E), threshold to increase forearm blood flow (37.09±0.22°C in P vs 37.17±0.26°C in E), slope of the sweating/esophogeal temperature relationship (0.42±0.16 in P vs 0.41±0.17 in E), or the slope of the forearm blood flow/esophogeal temperature relationship (10.04±4.4 in P vs 9.61±3.46 in E) were affected by 3 days of E_2 supplementation. In the animal study, rats received daily subcutaneous injections of either a vehicle (sesame oil; n=18) or estradiol (10 ug/100ml g b.w.; n=18). Within each group, 3 subgroups were utilized: a) 4-day, b) 8-day, or (c) 12-day treatment. Four hours after the final daily injection, rats underwent a heat tolerance test (HTT) consisting of treadmill exercise at 21.5 m/min at 35°C until colonic temperature (Tc) reached 40.4°C. Vehicle treatment had no effect on initial Tc, time to reach 40.4°C, or heating rate between treatments. However, initial Tc values were reduced, heating rates were lower, and times to reach 40.4°C were increased in rats treated with E_2 for 8 and 12 days compared with the 4-day treated group ($P<0.05$). Moreover, both initial Tc and heating rate were lower and time to 40.4°C was higher in E_2- vs vehicle-treated rats for both 8- and 12-day protocols. These results show that E_2 supplementation (a) has no effect on heat transfer to the skin or heat dissipation by evaporative cooling, and (b) can increase thermotolerance in ovariectomized rats exercising at high ambient temperature.

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II. Human Studies

A. Effect of estrogen supplementation on exercise thermoregulation in pre-menopausal females

1. Introduction. Several studies indicate that estrogen replacement therapy in post menopausal women not only reduces the physiological symptoms of estrogen withdrawal, such as the occurrence of hot flashes (20), but also reduces the incidence of cardiovascular disease (38). Interestingly, there is controversy over the thermal adaptive response of women, particularly related to the menstrual cycle. While some studies show no effect of sex hormones on adaptation to heat (14, 16, 18), more recent data demonstrate that estrogen therapy lowers the core temperature threshold for the increase in forearm blood flow (FBF) and sweating rate in post menopausal women exercising in a warm environment (41). Similarly, rats treated with estrogen display increased evaporative water loss at all levels of core temperature, they show a reduced core temperature threshold for the onset of saliva spreading, and they regulate their core temperature at a lower level during heat exposure (1).

Studies concerning the effects of estrogen on body temperature have not provided consistent findings. Previous studies have shown that estrogen supplementation decreases (9, 15, 41), has no effect (10, 21), or increases core body temperature (2, 3, 23). Silva and Boulant (36) found that estradiol (E_2) excited 26% warm sensitive neurons and inhibited 4% cold sensitive neutons in preoptic tissue slices. Such neuronal action would facilitate heat loss, reduce heat production, and therefore, decrease body temperature. This suggests that hypothermic effects of estradiol are due to direct actions of estradiol on preoptic neurons. In contrast, Cagnacci et al. (3) demonstrated that the hyperthermic role of endogenous opioid peptides is significantly increased by estrogen treatment in postmenopausal women. During estrogen administration the acute naloxone-induced opioid withdrawal reveals the role of endogenous opioid peptides in the control of body temperature and LH, suggesting that the central mechanisms regulating LH secretion and body temperature are both markedly influenced by estrogens through endogenous opioid peptides. However, Czaja et al. (6) demonstrated that after 3 days E_2 treatment, rectal temperature increased significantly at the depth of 4 cm, but had no significant effect on measurements taken at a depth of 8 cm. This data suggest that the effects of E_2 on rectal temperature may relate to changes in peripheral blood flow rather than a shift in core temperature.

At the cellular level, heat shock proteins (HSPs) are produced in response to heat stress and are the central component of acquired heat tolerance (25). HSPs are differentially regulated in males and females, and increases in HSP70 and HSP90 occur in ovariectomized female rats following estrogen therapy (27). Our laboratory was the first to show that HSP70 may be produced in humans exercising in the heat, and thus HSPs may be useful biomarkers of both thermal history and thermal tolerance (32).

Because of the important link between estrogen replacement conferring improved thermoregulatory responses in post-menopausal women and rats, and estrogen supplementation increasing HSP70 in ovariectomized rats, we sought to determine if estrogen supplementation would alter thermoregulatory responses in pre-menopausal women during exercise. Previous research indicates that an assay of HSP70 in blood mononuclear cells may be a useful biomarker to evaluate the potential relationship between estrogen supplementation and thermoregulatory responses in pre-menopausal women during exercise (32).

In an emergency, such as a rapid deployment of military personnel to a hot environment, a lowered core body temperature threshold for the onset of sweating, and increase cutaneous blood flow would be critically important. Women have lower sweating rates compared to men and may be at a disadvantage during work in hot, dry environments (18). There is evidence that an increased temperature threshold for sweating in the luteal phase of the menstrual cycle may be related to progesterone mediated changes in temperature regulation (12, 39). However, the effect of estrogen supplementation on thermoregulatory responses has not been studied in normally menstruating women, nor has a cellular mechanism been investigated. The cellular mechanism likely involves HSPs.

Thus, the purposes of this study were to determine whether short-term estrogen supplementation improves thermoregulatory responses in pre-menopausal women and whether HSP70 can be used as a biomarker to assess these responses. Because estradiol therapy has been shown to enhance HSP synthesis and improve thermoregulatory responses during exercise in the heat, we hypothesize that: 1) HSPs may serve as a convenient and rapidly assayed biomarker of thermal tolerance and therefore could be used to

develop therapeutic strategies to optimize temperature regulation in women; and 2) short term estrogen replacement therapy can improve thermoregulatory responses in exercising women.

To minimize the possible interaction of progesterone and to maximize differences in estradiol levels between estradiol supplemented and control subjects, this study was conducted with women in the early follicular phase of their menstrual cycle. In the follicular phase, estradiol levels average 40-80 pg/ml. A short-term estradiol supplementation protocol of 2 mg three times per day for 3 days, developed and tested for safety in *in vivo* fertilization programs, was used to elevate estradiol levels > 1000 pg/ml.

2. Methods. *Subjects.* Twenty-one females, aged 21-39, volunteered to participate in this study. Subjects were non-smokers with normal menstrual cycles, who refrained from oral contraceptive use for at least 2 months prior to the experiments. Each volunteer was informed of the testing procedures, and written consent to participate was obtained. Subjects were given a physical examination and experimental procedures were approved in advance by the Human Subjects Committee at the University of Iowa.

To identify the follicular phase, daily basal body temperature (BBT) was taken at least 1 cycle before the experiment to verify that the normal luteal elevation in BBT had occurred (12). In addition, serum estradiol and progesterone concentrations, were measured to verify day one of the menstrual cycle. Using a double-blind design, subjects were randomly assigned to either an estrogen supplementation (n=10) or a placebo (n=11) group. Experiments were conducted between November and December to preclude heat acclimatization. Age, VO_2 max, and body weight to surface area ratio were not significantly different between the two groups (Table 1).

Estimation of maximal and submaximal oxygen consumption. Before the experiment, each subject's VO_2 max was determined by an incremental cycle ergometer (CYBEX) test. Subjects completed three 3-min workloads of 75, 100, and 125 W. At the end of the last three min period, workload was increased by 25 $W \cdot min^{-1}$ each min until exhaustion. VO_2 andVCO_2 were analyzed continuously (Quinton Q-PLEX Cardio-pulmonary system), and heart rates were measured every min. A maximal test was defined by achievement of at least two of the following three criteria: a plateau or decrease in O_2 despite an increase in workload, a respiratory exchange ratio (RER) greater than or equal to 1.1, or attainment of at least 90% of predicted maximum heart rate (5). The workload required to elicit 60 % VO_2 max was determined from submaximal VO_2 values.

Study design. Each experiment consisted of a 5-min rest period followed by 20 min of cycling exercise at 60 % VO_2 max. Cycling was performed in an upright posture, in an environmental chamber maintained at 25° C, 30% RH. Subjects ingested 2 tablets of estrace on day 1, and 3 tablets on days 2 and 3 of their menstrual cycle. The last tablets were ingested 1-2 h prior to the experiment. All experiments were carried out between 6:30 a.m. and 9:00 a.m. On the first day of the subject's menstrual cycle, a blood sample was obtained to verify blood estradiol (E_2) and progesterone (P_4) concentrations, and for analysis of leukocyte heat shock protein 70 (HSP70). Additional blood samples were obtained prior to the experiment, and 6 h and 24 h following ingestion of the last tablet, for HSP 70 analysis. Serum Estradiol and progesterone concentrations were also measured on the 2nd blood sample.

Experimental protocol. Subjects were instructed to fast overnight and to refrain from heavy exercise for 24 h before the experiment. On arrival at the laboratory, subjects ingested 6 $ml \cdot kg^{-1}$ water to ensure hydration. A blood sample was obtained for HSP70 determination and for estradiol and progesterone measurement. Body height and weight were measured, and a heart rate monitor was placed around the chest at the level of the xyphoid process. Subjects then entered the environmental chamber and sat upright on the cycle ergometer for ≥ 45 min, allowing for instrument set-up and postural equilibration. An esophageal thermocouple, equal in length to 25% of each subject's height, was swallowed and placed at the level of the heart (42) to measure esophageal temperature (T_{es}). Copper-constantan thermocouples were placed on the upper arm, chest, thigh, and lower leg for skin temperature (T_{sk}) measurements (28). For FBF measurements, a double-latex cuff and two pressure cuffs were placed on the forearm, arm and wrist, respectively. A capsule for determining the local sweating response by resistance hygrometry was placed on the right forearm 2 cm below antecubital fossa.

Calibration procedures for FBF, T_{es}, T_{sk} and sweat rate (SR) measurement were conducted immediately prior to each experiment .

Measurement. Esophageal temperature was monitored with a YSI sensor (ESO-1) connected to a thermocouple transmitter (Tx-52-T_2). Changse in Tes were recorded on a Honeywell visicorder. Because swallowing saliva may lower T_{es}, subjects avoided swallowing by spitting saliva into a cup.

Mean skin temperature was calculated by a weighted sum of four different skin temperature measurements (28). T_{sk} was calculated as: upper arm (0.3) + chest (0.3) + thigh (0.2) + lower leg (0.2). Skin temperature data were collected by IBM computer using datalog software. Heart rate (HR) was measured with a heart rate monitor (Polar Electro).

Venous occlusion plethysmography was used to measure FBF on the left arm. During the experiment, the left hand was excluded from the circulation with a pneumatic cuff at the wrist inflated to 180-220 mmHg. A second cuff placed around the upper arm was inflated to 50 mmHg twice per min (15 seconds on and off interval). FBF is computed from the slopes of plethysmographic curves recorded continuously on the visicorder. FBF measurements were obtained with the forearm extended horizontally and placed above venostatic level.

Local sweating rate of the right forearm was determined from a continuously ventilated lithium chloride sensor within a 6.605 cm^2 capsule. Flow rate was adjusted to optimize sensitivity and to ensure adequate data range for the recorder. Calibration of the hygrometer system involves determining the trace deflection from baseline for a given change in air flow through the humidification system. A regression equation is determined for the relationship between trace deflection and saturated air flow. Sweat rate was calculated from the regression line by converting rates of air flow through the humidification system into equivalent sweat rates.

Tes, FBF, and SR data were recorded on a Honeywell Visicorder. T_{es} and SR were measured continuously throughout the experiment, FBF was measured every 30 s, T_{sk} and HR were measured every min. The threshold was defined as the point after which a continuous increase in FBF occurred. The slope of the linear regression relating the rise in FBF and SR to Tes was used to characterize the sensitivity of the skin blood flow and sweat rate responses.

HSP analysis. Human leukocytes were isolated at room temperature (22° C) by adding 5 ml of Ficoll-Hypaque to the blood (10 ml) and centrifuged (3000 rpm, 25 -30 min). Leukocytes were collected and then washed (centrifugation at 1,500 rpm for 5-7 min) twice with phosphate buffer solution (PBS). The final leukocyte pellet was suspended in 0.25 ml PBS, and stored at -70° C until analysis. This isolation procedure was initiated immediately on attainment of a blood sample. HSP70 analysis was performed as previously described using western blot analysis (32). Serum progesterone and estradiol concentrations were measured using a radioimmunoassay (13).

Statistic analysis. Significant differences ($P < 0.05$) were calculated using a two - factor analysis of variance (ANOVA) for repeated measurements and Scheffe test. Values presented are mean ± SE.

Linear regression analysis was employed for each experiment to obtain thresholds and slopes of the sweat rate vs. esophageal temperature and forearm blood flow vs esophageal temperature relationships.

3. Results. Serum estradiol concentration was unaffected by placebo treatment, but was significantly elevated ($P < 0.05$) after estrogen supplementation (Table 2). Serum estradiol concentration were similar ($P > 0.05$) between the two groups before estrogen supplementation, and after placebo treatment. Serum progesterone concentration was not significantly different between the two groups or between pre and post-estrogen supplementation ($P > 0.05$).

Heart rate (HR), mean skin temperature (MST), and esophageal temperature (T_{es}) responses during exercise were not significantly different between estrogen supplementation and placebo groups (Fig. 1). MST was lower in the estrogen supplementation group than the placebo group, but the difference was not significant. Both groups showed a fall in MST at the onset of exercise, then an increase as Tes rose. The rate of rise in MST, T_{es}, and heart rate during exercise was not affected by estrogen supplementation.

FBF and SR responses during exercise were not significantly different between groups (Fig. 2). Tes threshold for SR and increase in FBF, slope of T_{es}-SR, and T_{es}-FBF relationships were plotted, each line represents the data on a representative subject (Fig. 3A, 3B). There were no significant differences between groups (Table 3).

Heat shock protein synthesis was not induced by 3 days of estrogen supplementation (Fig. 4). There were no significant differences between first (baseline), second (1 h after last tablet), third (6 h after last tablet), or fourth (24 h after last tablet) blood samples in a given group or between groups (Fig. 4).

4. Discussion. The primary contribution of this study is that it provides evidence indicating that 3 days of estrogen supplementation (ES) during the early follicular phase of the menstrual cycle of pre-menopausal female subjects has no effect on thermoregulatory responses. Tes thresholds for the initiation of sweating and increase in FBF were not altered by estrogen supplementation. Tes also was not significantly different between groups during rest or after 20 min of cycle exercise. SR, MST, HR and FBF were similar between the two groups throughout the exercise. Furthermore, HSP70 synthesis was not induced by 3 days of estrogen supplementation.

Effect of ES on core body temperature. Previous studies have shown that ES decreases (9, 15, 41), has no effect (10, 21), or increases core body temperature (2, 3, 23). In this study, core temperature was not changed by ES. Israel and Schnell (15) demonstrated that a single intramuscular injection of 1.5 mg of estradiol benzoate in 5 regularly menstruating women lowered rectal temperature during the postovulatory phase of the menstrual cycle. Using postmenopausal women, Tankersley et al. (41) reported that 14-23 days of estrogen replacement therapy significantly lowered resting Tes and rectal temperature, and lowered the Tes thresholds for the initiation of sweating and increase in FBF during exercise in a warm environment (36°C, 27.5% RH) (41). Baker et al. (1) found similar results in ovariectomized rats exposed to a 38°C environment after 9 weeks of estrogen replacement (0.25 mg subcutaneous implant) (1). Although at the beginning of the experiment body core temperature was not significantly different between E_2 treated and untreated animals, E_2 treated animals increased evaporative water loss at all levels of core body temperature and reduced the core body temperature threshold for the onset of saliva spreading (1). Fregly et al. (9) also reported that 21 weeks of subcutaneously implanted E_2 treatment (36 μg/kg per day) lowered rectal temperature in rats kept in a 26°C environment. Cagnacci et al. (3) found that the hyperthermic effect of endogenous opioid peptides was significantly increased by estrogen treatment (transdermal E_2, 50 μg/day for 2 months in post menopausal women) in a 22°C environment. This stimulatory effect of estrogen was not associated with an increase in baseline body temperature, but was most likely responsible for the increased activity of counter regulatory neurotransmitters, which act to maintain a constant body temperature (2).

On the other hand, Frye et al. (10) showed that rectal temperature was not significantly different among preovulatory, postovulatory, and amenorrheal women before and after acclimation to a hot, dry environment (Tdb/Twb = 48/25°C), suggesting that estrogen plays no role in resetting core temperature. Laudenslager et al. (21) showed that 7 days of E_2 treatment via subcutaneous Silastic capsules in ovariectomized rats tested at 2.5, 10, 20, and 30°C did not affect colonic temperature. In contrast, Marrone et al. (23) found an elevation in rectal temperature after 17 days of injecting E_2 subcutaneous (1, 3, or 10 μg) into ovariectomized rats in a 24°C environment. This rise in colonic temperature was inversely related to E_2 dosage.

These different findings are attributed, in part, to the following factors: a) The conditions of the subjects. Pre-menopausal, post-menopausal, and ovariectomized animals have served as experimental subjects. b) Phase of the menstrual cycle. It has been demonstrated (8, 12) that body temperature is regulated at a higher level during the luteal than during the follicular phase of the menstrual cycle in women with normal periods. Controlling the time of day experiments are performed and menstrual cycle phase are as important as controlling for aerobic power, age, and fitness in studying female thermoregulatory responses during exercise. c) Dosage of estrogen employed. Esrael and Schneller (15) injected subjects with 1.5 mg E_2 compared with 18 mg given orally for 14-23 days (41) or 3 days in the present study. d) Type of estrogen supplementation. Information comparing different estrogen formulations with regard to blood vessel effects and temperature regulation are not available (35). And e) Different environmental conditions. Thus, the effect of estrogen supplementation on temperature regulation in women needs further investigation.

Effect of ES on FBF. Van Buren et al. (43) demonstrated that administration of L-nitroarginine methyl ester (L-NMMA), a competitive inhibitor of nitric oxide synthase, to ovariectomized ewes reduced uterine blood flow. This attenuation of estrogen-induced flow in the presence of L-NMMA suggests that the vasomotor effects of estrogen may be related to endothelial production of nitric oxide (43). Volterrani et al. (44) reported that in post-menopausal women, FBF was significantly greater in the E_2 treatment group than in the placebo group 40 min after administrating 1 mg of E_2 sublingually. Acute E_2 administration (iv ethinyl estradiol, 35 μg in 15 min) enhances the coronary vasodilator response to the endothelium-dependent vasodilator acetylcholine in post menopausal women (29). Acute (20 min) intra-arterial E_2 administration enhanced endothelium-dependent vasodilation in the forearms of post

menopausal women, including those with evidence of pre-existing vascular dysfunction (11). After chronic (3 weeks) transdermal E_2 administration (0.1 mg/day), however, which achieved a plasma E_2 level of 120 ± 57 pg/ml, the vasodilator responses to acetylcholine and to sodium nitropruside were unchanged from control values. In oophoretomized monkeys, however, both acute (20 min, i.v.) and chronic (2 years, oral) estrogen administration improved acetylcholine-induced coronary vasodilation (47, 48). In the present study, estrogen supplementation had no effect on FBF among pre-menopausal women (Fig. 2).

Evidence from these studies suggests that acute (within 60 min) estrogen supplementation elevates blood flow in several vascular beds in both post-menopausal women and ovariectomized animals. The mechanism lies in the production of nitric oxide from endothelial cells (4). Estrogen is known to have antioxidant properties (33) and oxidized lipids reduce nitric oxide activity (37). Estrogen may influence vascular responses by reducing lipid peroxidation in the arterial wall, thereby allowing more nitric oxide to reach vascular smooth muscle. Long term estrogen supplementation (14-23 days) in the study by Tankersley et al. (41) could have generated tolerance to the effect of E_2 on the endothelium-dependent vasodilation. Long term estrogen supplementation acts through a different cellular mechanism and includes regulation of gene expression, which opposes the acute potentiation of endothelium-dependent vasodilation (11). It is still possible that acute ES can increase vasodilation in pre-menopausal women because in this study 3 days of estrogen supplementation is considered a long term effect of estrogen supplementation relative to other studies (within 60 min). However, evidence suggests that blood vessel integrity is well protected in pre-menopausal women, and estrogen may protect the integrity of blood vessels by one of several mechanisms. The hormone may inhibit the release of a vasoconstrictor substance such as endothelin (24). Estrogen decreases lipoprotein induced smooth-muscle proliferation (7) in blood vessels and may inhibit intimal proliferation associated with mechanical injury to endothelium (30, 31, 45). In post-menopausal women, steroidogenesis by the follicle and corpus luteum is impaired and the neuroendocrine balance is disturbed. Thus, blood vessel integrity is not protected.

Effect of ES on SR and skin temperature. In the present study the slope of the Tes-SR relationship was nearly identical in the estrogen supplementation and placebo groups. This agrees with the work of other investigators (8, 40). Frye et al. (10) also reported the absence of a lower SR in the luteal phase of menstrual women suggesting that estrogen does not inhibit sweating. This is supported by hormone data on twins (one with a normal menstrating women, the other with amenorrhea). Plasma [E_2] was significantly higher in the menstruating twin, but no differences were apparent between the twins in sweating rate (10). In normal menstrual cycle without estrogen supplementation, however, Kawahata's (17) demonstrated that there were rather large changes in the latent period of thermal sweating (time required to start sweating) measured at different times during the menstrual cycle. At the time of menstruation, the latent period was much shorter than near the time of ovulation. He was able to lengthen the latent period of sweating in males by administration of estradiol (17). Sargent and Weinman (34) repeated Kawahata's (17) experiments on females at different times of the menstrual cycle, but they reported that sweating onset did not vary as a function of menstrual cycle phase. Physiological individuality was suggested by Sargent and Weinman (34) to explain the different results between these two experiments.

Skin temperature varies as a function of ambient temperature, and the effect of local skin temperature on the sudomotor (26) and vasomotor (46) responses to exercise are well known. In this study, the E_2 treated group had a lower T_{sk} throughout the experiment, but no significant difference was found between groups at any time (Fig. 1). Both groups showed a fall in MST at the onset of exercise followed by an increase as Tes rose.

When SR and FBF are expressed as a function of core temperature, an upward shift of Tes threshold is observed for the initiation of vasodilation and sweating during the luteal phase compared with the follicular phase of the cycle during exercise in the heat (8, 19, 39, 40). The magnitude of this shift corresponds with the difference in core temperatures observed between the two phases. Because Tes was not changed during rest by estrogen supplementation in the present study, Tes threshold for initiation of cutaneous vasodilation and SR were not changed.

Effect of ES on HSP70 synthesis. The HSP70 family is essential for cellular survival of heat stress, and its induction by non-heat stress or by genetic manipulation is sufficient to protect cells from thermal injury (25). In the present study, 3 days of estrogen supplementation did not induce of HSP70 synthesis. This may be attributed to the tissue specificity of the HSP70 response (25). E_2 significantly elevated HSP70 and HSP90 protein concentrations in the ventromedial hypothalamus of gonadectomized adult female rats (27). It was possible that the leukocytes used in the present study are not tissue specific to induce HSP70 synthesis by estrogen supplementation. Another factor to be considered is that HSP70 is

very sensitive to heat stress. Ryan et al (32) showed that HSP70 synthesis was only induced when T_{re} was elevated above 40°C in human subjects. Locke et al. (22) demonstrated that synthesis of HSP72 was induced in lymphocytes, spleen cells and soleus muscle after 20 min of exercise while rectal temperature elevated above 40°C. However, in this study, Tes only reached 38.1°C this temperature was too low compared to Ryan's studies in order to induce HSP70 synthesis.

In conclusion, this study demonstrates that 3 days of estrogen supplementation has no effect on core temperature and on thermoregulatory responses in young women during the follicular phase of their menstrual cycle. Regarding HSP70, it is importanbt to note that we have not observed a divergence in heat tolerance and HSP70 levels.

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6. Tables

Table 1. Subject chacteristerictics

Treatment	N	Weight (kg)	Height (cm)	Age (y)	VO_2 max (ml/kg/min)	BW/SA
ES	10	63.6 ± 2.2	166.1 ± 2.0	25.8 ± 1.8	38.4 ± 2.0	37.30 ± 2.70
PLACEBO	11	59.0 ± 2.8	166.2 ± 1.8	26.4 ± 1.8	37.1 ± 1.9	35.58 ± 2.85

Data are expressed as mean ± SE.

Table 2. Serum estradiol and progesterone concentration

	Placebo Pre	Placebo Post	ES Pre	ES Post	Follicular Phase	Luteal Phase
Estradiol (pg/ml)	34.8 ± 2.4	42.5 ± 4.2	32.55 ± 3.6	1166.6 ± 114*	20-60	150-600
Progesterone (ng/ml)	0.86 ± .07	1.02 ± .12	0.98 ± .11	.99 ± .14	0.1-1.5	7-25

* Significantly different from ES pre and pre and post placebos trials.

Table 3. Slope and T_{es} threshold of FBF and SR responses

Treatment	FBF threshold (°C)	FBF slope	SR threshold (°C)	SR slope
ES	37.17 ± 0.08	9.61 ± 1.10	36.97 ± 0.05	0.41 ± 0.05
Placebo	37.09 ± 0.08	10.04 ± 1.33	36.90 ± 0.07	0.42 ± 0.05

Data expressed as mean ± SE

Slope is expressed in ml•100 ml tissue^{-1}•min^{-1}•°C^{-1} and mg•cm^{-2}•min^{-1}•°C^{-1} for FBF and SR, respectively.

7. Figure Legends

Figure 1 Heart rate (HR), mean skin temperature (MST), and esophageal temperature (T_{es}) responses during placebos and estrogen supplementation (ES) trials, respectively. Data are as mean ± SE; n=10, and 11 in ES, and placebo groups, respectively.

Figure 2 Mean forearm blood flow and mean sweat rate as a function of T_{es}, standard errors were deleted for clarity. FBF and SR increase with increment of T_{es}. There were no significantly different between two groups throughout the experiment. N= 10, 11, in ES and placebo group, respectively.

Figure 3 T_{es} threshold for the increase of FBF (A) and SR (B), and slope of T_{es}-FBF, T_{es}-SR relationship were plotted. Each line represent 1 subject from each group.

Figure 4 Heat shock protein 70 synthesis following 3 days of estrogen supplementation. Data are presented as percent of the initial blood sample. Samples 1, 2, 3, and 4 represent blood samples collected before ES or placebo treatment, 1-2 hr after consuming the last tablet (just before experiment began), 6 hr after the last tablet was taken, and 24 hr after last tablet was taken, respectively. There were no significant differences among samples or between groups. Data represent as mean ± SE.

Fig. 1

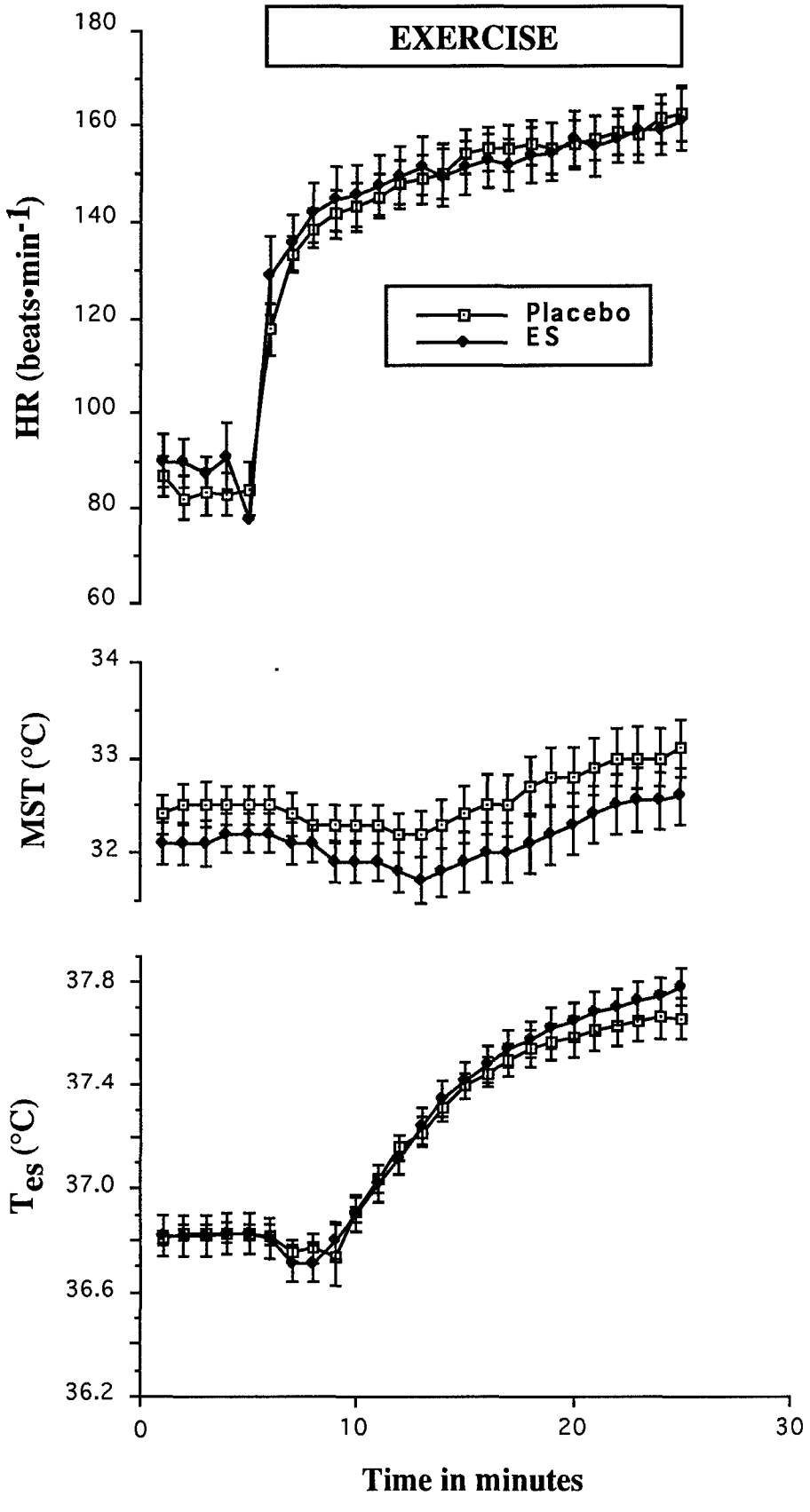

EXERCISE
HR (beats•min-1)
180
160
140
120
100
80
60
Placebo
ES
MST (°C)
34
33
32
Tes (°C)
37.8
37.4
37.0
36.6
36.2
0
10
20
30
Time in minutes

Fig. 2

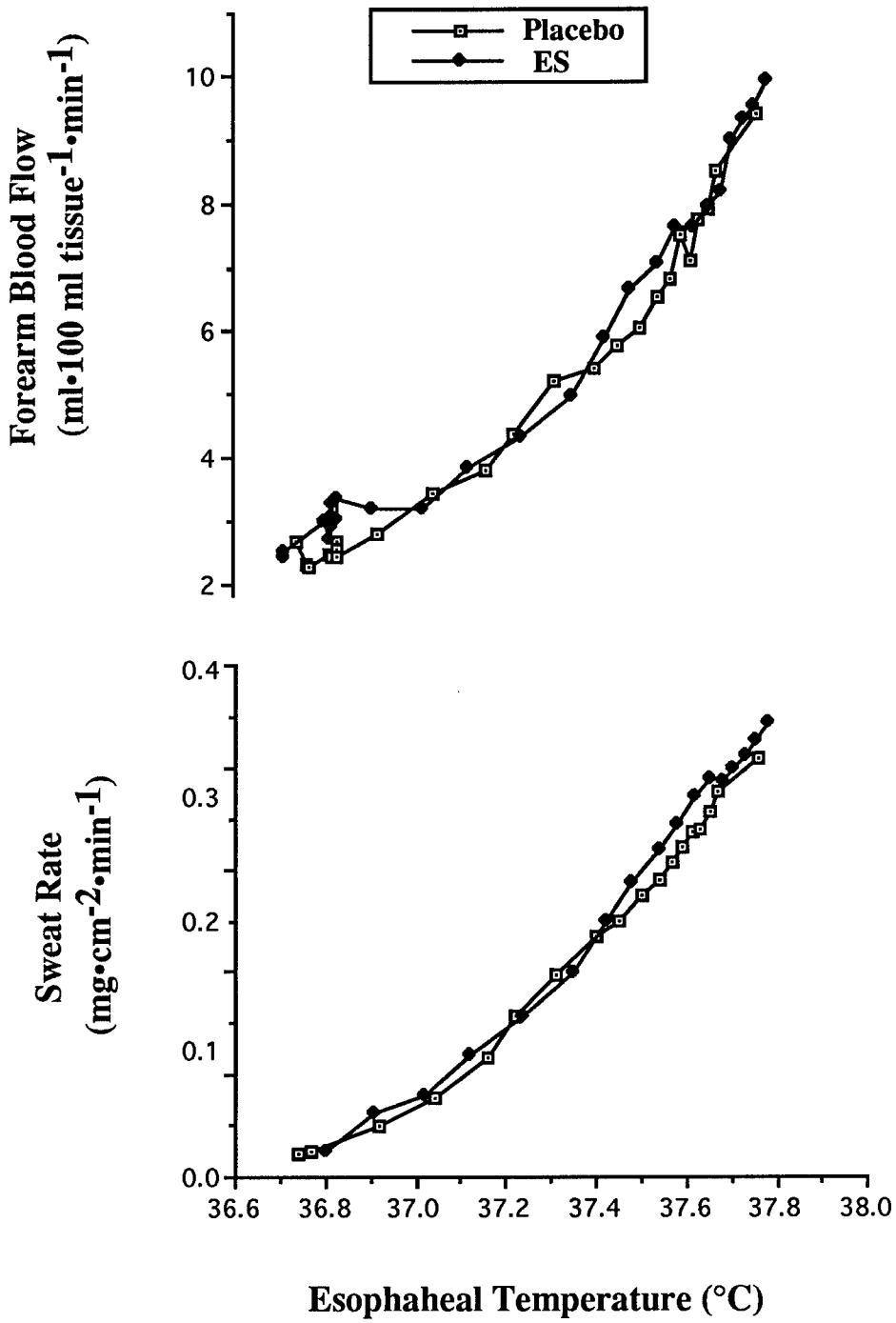

Placebo
ES
Forearm Blood Flow
(ml•100 ml tissue-1•min-1)
10
8
6
4
2
Sweat Rate
(mg•cm-2•min-1)
0.4
0.3
0.2
0.1
0.0
36.6
36.8
37.0
37.2
37.4
37.6
37.8
38.0
Esophaheal Temperature (°C)

Fig. 3

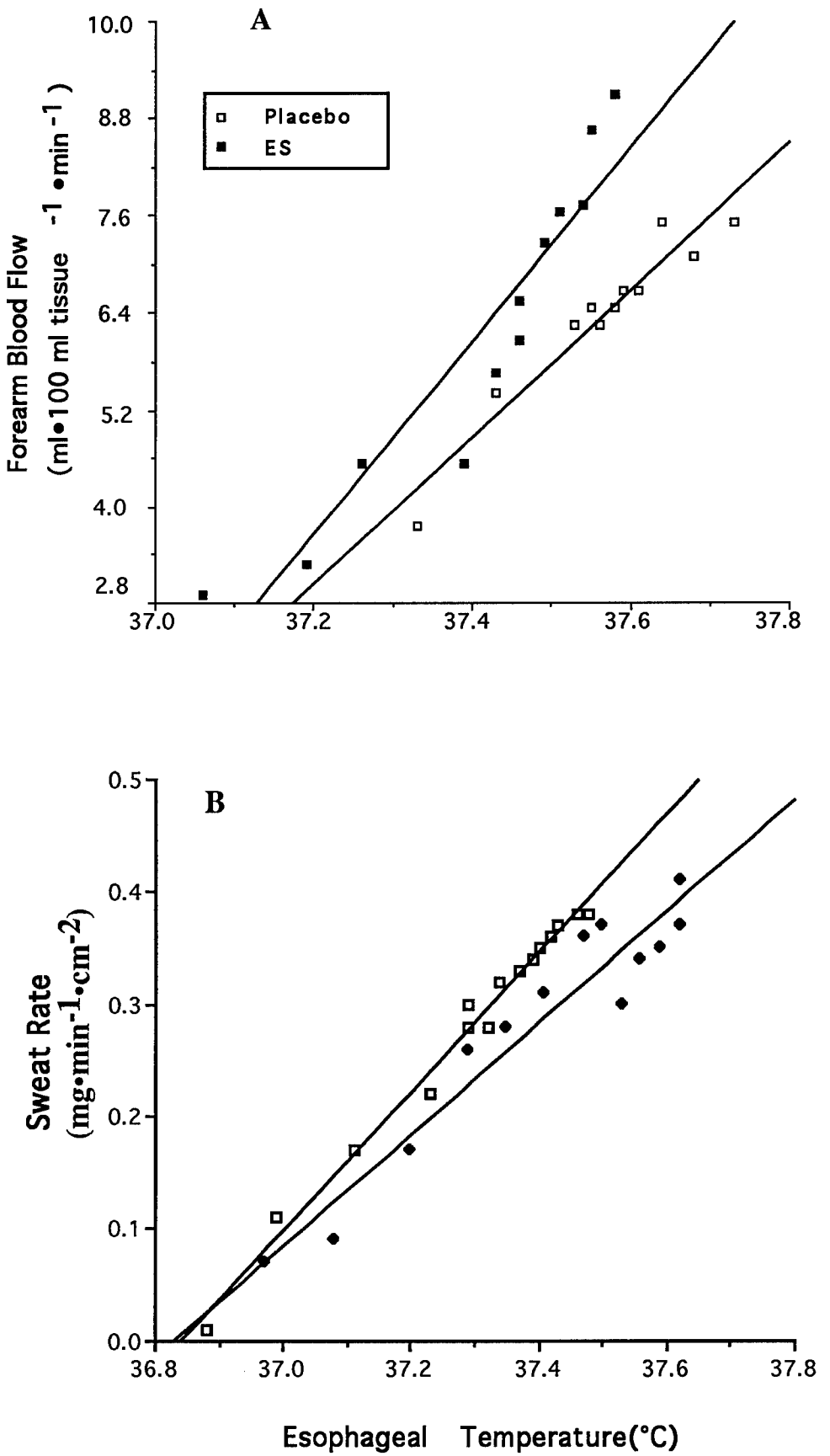
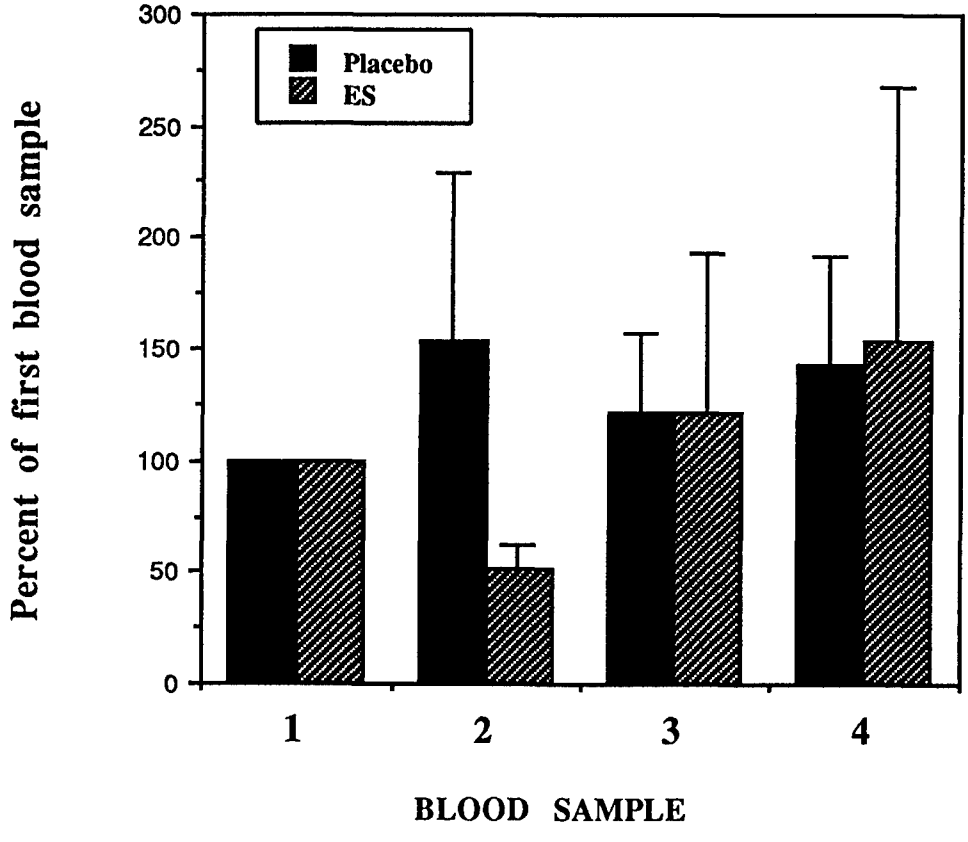

A
Placebo
ES
Forearm Blood Flow
(ml•100 ml tissue -1 •min -1)
10.0
8.8
7.6
6.4
5.2
4.0
2.8
37.0
37.2
37.4
37.6
37.8
B
Sweat Rate
(mg•min-1•cm-2)
0.5
0.4
0.3
0.2
0.1
0.0
36.8
37.0
37.2
37.4
37.6
37.8
Esophageal Temperature(°C)

Fig. 4


Placebo
ES
300
250
200
150
100
50
0
Percent of first blood sample
1
2
3
4
BLOOD SAMPLE

II. Animal Studies

A. Effects of estradiol on thermotolerance during exercise and heat stress

1. Introduction. Over the past decade, there has been a dramatic increase in the number of women participating in competitive, strenuous sports activities. This has lead to a parallel rise in research devoted to the exercise responses and adaptations observed in women. In particular, it has become apparent that sex differences in temperature regulation and heat tolerance exist in humans (22). Furthermore, there are conflicting reports regarding the thermal adaptive response of women, especially related to the menstrual cycle. Therefore, it is of considerable interest to investigate the mechanisms associated with heat adaptation responses observed in women .

Very little work has focused on the influence of the hormonal cycle on exercise performance. Given that estrogens and progestins can have individual or interactive effects on a variety of metabolic processes, the potential exists for an influence on athletic performance. The role of estrogen in body temperature regulation has only recently been addressed in post-menopausal women. It has been demonstrated that estrogen is significant in maintaining proper body temperature regulation in post-menopausal women, reducing hot-flashes in addition to increasing heat dissipatory mechanisms during exercise (34). These data suggest a beneficial role of estrogen thermoregulatory responses in post-menopausal women.

Due to the link that has been established between an improvement in exercise performance in the heat as a result of hormone replacement therapy in the form of estradiol, and estradiol supplementation increasing plasma volume and thermoregulatory mechanisms, we postulate that estradiol will enhance exercise performance in a warm environment. Our specific purpose is to test the hypothesis that estrogen therapy in ovariectomized female rats is associated with enhanced thermotolerance to exercise in the heat. The unique contribution of these experiments lies in our methodology and approach to the problem. Hyperthermia is a physiological stress regularly experienced by a majority of our population. It is noninvasive and does not make use of exogenous pharmacological manipulation. Moreover, a variety of questions including hormonal contributions to thermoregulation and exercise performance in the heat and cellular stress responses may be addressed. The rat is a useful animal for studying the mechanisms of action of estrogen on thermoregulation in heat stress, and can be utilized to define the upper limits of temperature exposure and the tissue specificity of the response. The information derived from these studies may in turn be an indication of therapeutic interventions for athletes competing in the heat.

2. Methods.

Animals. Two groups of 8 week old female Sprague-Dawley rats (Harlan, Madison, WI) were utilized to conduct these experiments: a vehicle control group (251-300 g, n=18) and an estradiol treated group (220-263 g, n=18). Ovariectomized rats were used to precisely control for the effects of female reproductive hormones on the HSP response. OVX rats have no estrous cycle and no circulating plasma estrogen and progesterone, thereby enabling experimental manipulation of hormone concentrations. Ovariectomies were performed by the supplier prior to shipment. Rats were housed in group cages in a temperature-controlled animal facility with a 12:12-h light-dark cycle and were provided standard rat chow and water ad libitum. Experiments and animal care procedures were performed in accordance with institutional guidelines. All rats were familiarized with the testing environment and a colonic thermistor probe several times over the week preceding an experiment. Animal weights were recorded immediately before and after each experiment.

Experimental Protocol. Within each of the two groups, animals were randomly divided into three sub-groups each with a separate protocol dependent upon the duration of injections: 1) Four day treatment protocol, 2) Eight day treatment protocol, and 3) Twelve day treatment protocol. Each injection was administered by one investigator at the same time of day to ensure consistency of hormone treatment throughout the protocol. The vehicle control group was administered a 0.1 ml sesame oil/100 g body weight dose and the estradiol treated group was given a pharmacological dose of 17 13-estradiol 3-benzoate (Sigma Chemical, St. Louis, MO): 10 μg •0.1 ml of sesame oil^{-1} · 100 g body weight^{-1} injected into the subcutaneous dorsal neck skinfold. Four hours following the final daily injection for each protocol, the rats were prepared for the experimental intervention. All experiments were conducted on conscious unrestrained rats. Internal body temperature was monitored continuously during each experimental protocol by a thermistor probe inserted 6-7 cm beyond the anal sphincter into the colon (T_c). Following attachment of the probe, each rat was placed inside an enclosed small animal treadmill unit maintained at an ambient temperature (T_a) 35°C.

The rats were exposed to an exertional heat tolerance test (HTT) consisting of continuous treadmill exercise at a velocity of 21.5 m/min (0% grade). The experimental protocol was terminated when the animals attained a T_c =40.4°C. This Tc was chosen because it was demonstrated in the rat that T_c values of 41.0 and 40.4°C are the minimal lethal temperatures for death due to nonexertionally and exertionally induced heat exhaustion, respectively (15). Upon completion of the exercise challenge, the rats were removed from the treadmill unit and placed into their home cage for four hours prior to collection of tissue samples. The rationale for choosing this time point was that previous data indicate that maximal HSP70 induction to estradiol occurs in this 4-12 hour post-injection period (23, 27, 31). In general, the heat tolerance test was administered four hours following the last injection of either estradiol or vehicle.

Assays. Following the four hour waiting period to allow adequate time for protein synthesis, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). It has been shown that this procedure will have no effect on HSP70 synthesis (5). A midline incision was made on the ventral surface of the rat. A 2 ml sample of heparinized blood was obtained via exsanguination through the inferior vena cave and placed into a bullet tube for centrifugation. Blood samples obtained during exsanguination were used for measurement of hematocrit, and plasma osmolality. Hematocrit was measured in duplicate immediately after bleeding using microhematocrit tubes and a microcentrifuge. The blood samples were centrifuged for six minutes and the ratio of red blood cells to total blood volume was determined. For the measurement of plasma osmolality, the heparinized, whole blood sample was centrifuged immediately after collection and aliquots of plasma were stored at -70°C. The aliquots were then thawed and analyzed for plasma osmolality using a vapor pressure osmometer.

Analysis. There were 36 ovariectomized female rats separated into two treatment groups consisting of a vehicle control group and an estradiol treated group. These groups were in turn divided into three sub-groups depending upon the duration of the treatment protocol. All results are presented as means + SE. Appropriate statistical analysis was performed to determine significant differences in animal weight (pre- vs. post-Heat Tolerance Test), initial and final T_c values, heating rate, and heating rate as a function of body mass. In addition, hematocrit and plasma osmolality were also analyzed. Significant differences were determined with an analysis of variance for two factors design (Comparison between protocols within each treatment group and comparison between treatment groups within a given protocol) followed by Duncan's post hoc analysis. Significance was established at the $P < 0.05$ level.

3. Results. As shown in Table 1, the administration of sesame oil vehicle had no effect on either the initial T_c or the change in T_c during the heat tolerance test within any of the three protocols. In addition, there were no significant differences among the three vehicle-treated groups with respect to heating rates and the length of time to reach 40.4°C.

The administration of estradiol for eight and twelve days had significant effects on baseline body temperature (Table 1). The eight day and twelve day estradiol-administered animals had significantly lower initial T_c values when compared to the four day treated group ($P<0.05$). A reduction in resting core temperature was also demonstrated in middle-aged women undergoing estrogen replacement therapy, suggesting that estrogen acts centrally to lower the set point about which T_c is regulated (34). Therefore, the change in T_c to reach 40.4°C was significantly elevated in both the eight and twelve day estradiol-treated animals. In addition, the length of time to reach 40.4°C was significantly increased in the eight and twelve day estradiol-treated protocol. The heating rate and heating rate as a function of body mass was also significantly lower in the eight and twelve day treated animals when compared to the four day treated estradiol protocol. Furthermore, the eight and twelve day estradiol-treated groups displayed a significantly lower initial T_c when compared to the vehicle-treated animals undergoing the similar time-matched treatment protocol (i.e., 8 day estradiol vs. 8 day vehicle and 12 day estradiol vs. 12 day vehicle). The change in T_c and the time to 40.4°C in the eight and twelve day estradiol-administered animals were significantly higher than the time-matched vehicle-treated animals within the comparable protocol. In addition, estradiol administration significantly reduced the heating rate and heating rate as a function of mass in the eight and twelve day protocols when compared to animals treated with sesame oil vehicle for the same duration of time. The four day estradiol-treated animals had a significantly elevated heating rate and heating rate/mass when compared to the four day vehicle-treated animals.

Vehicle-administered animals within the eight and twelve day protocols had significantly elevated body weights both before and after the heat tolerance test when compared to the four day vehicle-treated group (Table 2). Body weight was lower in estradiol-treated animals, an effect of estrogen that is commonly observed in ovariectomized rats (2, 20). Both the pre- and post-weight measurements within each of the three protocols in the estradiol-treated animals were significantly reduced when compared to animals

undergoing sesame oil vehicle treatment for the same duration of time. Furthermore, the body weight measurements obtained prior to the exercise intervention were significantly lower in the eight and twelve day estradiol-treated animals when compared to the four day estradiol-treated group. Estradiol treatment significantly decreased body weight following the heat tolerance test in the eight day protocol compared to the four day protocol. Animals administered estradiol for twelve days displayed a trend for a reduction in post-weight measurements when compared to the four day estradiol-treated animals ($P<0.10$). However, the administration of estradiol for any of the three protocols had no effect on the change in body weights of the animals during the heat tolerance test. Estradiol administration had minimal effects on hematocrit and plasma osmolality (Table 2). There was a tendency for hematocrit to be lower in estradiol-treated animals when compared to each protocol's time-matched vehicle-treated animals. However, this difference was only significantly in the four day estradiol-treated group. Furthermore, a trend for an elevated plasma osmolality was observed in the estradiol-treated animals with respect to the time-matched vehicle-treated animals. Again, this was only significant during the four day estradiol treatment protocol.

4. Discussion. The effects of hormonal therapy in females exercising in the heat has only recently been addressed. To study the effects of estrogen in regulating core temperature, several investigators have examined the consequences of exogenously administered estrogen in middle-aged women exhibiting menopausal symptoms. This approach is utilized because estrogen withdrawal during menopause has been associated with signs and symptoms that are analogous to heat loss responses (19). Specifically, the sensation of hot flashes coincides with increased skin blood flow and sweat rate (19). The onset of heat loss responses has been demonstrated to be lower in middle-aged women administered estrogen replacement therapy during exercise exposure in a warm environment, thereby reducing the thermoregulatory strain (34). This was determined by a decrease in the threshold body temperature for the onset of both sweating and cutaneous vasodilation, measured via forearm blood flow (34). It is suggested that the elevated plasma estrogen concentration acts centrally to lower the set point about which T_c is regulated at rest and during a heat and exercise challenge (34). The mechanisms of these effects of estrogen during heat stress are not known and could involve direct or indirect actions on peripheral effectors or on central nervous thermoregulatory control regions. In addition, these effects could be secondary either to systemic actions involving alterations in body fluid balance, or to the cellular contributions to thermotolerance via heat shock proteins. It was the purpose of this study to measure the effects of chronic estrogen replacement throughout three different time courses of therapy on thermotolerance in ovariectomized rats exercising in a hot environment. Not only did we evaluate estrogen's effects on thermotolerance, but also we delineated a time course of action of estrogen therapy on heat loss mechanisms.

The present experiments demonstrated that plasma estrogen levels obtained through both the eight and twelve day treatment protocols had a marked effect on thermotolerance in ovariectomized rats exercising at a high ambient temperature. Estrogen-treated animals within the eight and twelve day groups had a lower heating rate, and the length of time that was necessary to elevate T_c to 40.4°C was significantly increased when compared to the four day estradiol treated animals, in addition to the time-matched vehicle-treated animals for each protocol. Furthermore, estrogen administration for the eight and twelve day groups resulted in significantly reduced resting colonic temperatures compared to the four day estradiol-treated animals and each group's time-matched vehicle-treated animals, indicating that estrogen acts centrally to lower the set point about which body temperature is regulated in ovariectomized rats. This is indicative of a time course of estrogen administration that is required to enhance thermotolerance in ovariectomized rats, as measured by the animals ability to maintain exercise in the heat for a significantly longer duration of time. further, because there were no statistical differences observed between the eight and twelve day estradiol-treated groups, it is suggested that estrogen's effects on thermotolerance reach a plateau within eight days of hormone therapy.

The enhanced thermotolerance in the eight and twelve day estradiol-treated animals could be a function of the significantly reduced body weight observed when compared to vehicle-treated animals. Because we utilized the same absolute workload in all the animals without assessing VO_2 max, the decrease in body weight would allow the animals to run longer to perform the same amount of work as heavier vehicle-administered animals (18). The reduced body weight would enable the animals to be exercising at a lower intensity, or lower percent of VO_2 max, when compared to the heavier oil-treated animals, which would contribute to the significant increase in the exercise duration throughout the heat tolerance test. Kendrick et al. (17) suggested that the differences in body weight may possible affect the recruitment of muscle fibers and alter the muscles' pattern of fuel use while the same amount of work has been performed. However, this may not be a critical factor to the enhancement of thermotolerance in this study because when normalized

for body weight, the animals within the eight and twelve day estradiol groups still displayed a significant reduction in heating rate as a function of mass.

Body Temperature Regulation. Enhanced thermotolerance in ovariectomized rats could be related to the influence of estrogen on thermoregulation in heat stress. The effects of estrogen replacement on thermoregulatory evaporation and body core temperature in ovariectomized rats resting in a hot environment have been previously evaluated (2). It was demonstrated that the rate of evaporation at a given core temperature for estrogen-treated animals was higher during the passive heat exposure period, and the threshold core temperature at which the estrogen-treated animals initiated spreading saliva was lower than untreated animals (3). Evaporative water loss was utilized to measure thermoregulatory responses because the rat uses saliva spreading for evaporative cooling (33). Therefore, administration of estradiol to ovariectomized rats in the present study should enable the animals to maintain a lower colonic temperature during an exercise challenge in the heat. Moreover, evaporative water loss should be higher at any given core temperature after estrogen replacement therapy due to a lower core temperature threshold for the onset of heat loss responses. This would allow the animals to sustain exercise in a warm environment for a longer duration of time, as was observed during the heat tolerance test in the eight and twelve day estradiol-treated groups. In addition, similar effects of estrogen on body temperature and on evaporative cooling have been observed in human females during exercise in a warm environment (34). Baker et al. (2) suggest that central neural regions controlling body temperature could be mediating the influence of estrogen on thermoregulatory evaporation, indicated through the similarity of the hormonal therapy on heat loss mechanisms in two difference species, each possessing distinct methods of evaporative heat loss. The hypothalamus is involved in regulating appetite, thirst and water conservation, temperature, sleep, autonomic balances, and endocrine functions (12). Specifically, due to the presence of thermosensitive neurons in the preoptic anterior hypothalamus, this region of the brain plays a significant role in thermoregulation (4). When brain slices of the preoptic area of the hypothalamus in the rat were bathed in an estradiol (E2) perfusate, 26% of the warm-sensitive neurons responded with an increased firing rate (32). This result suggest that E2 can stimulate a greater heat loss response in the rat, and its site of stimulation may involve the preoptic hypothalamic neurons that are known to regulate body temperature (32). However, preoptic estrogen implants had no effect on T_c of rats resting in a euthermic environment at an ambient temperature of 24°C. In addition, normal fluctuations of the hormonal environment during the menstrual cycle may influence thermoreuglatory responses during exercise and heat stress. Both estrogen and progesterone levels are elevated during the post-ovulatory luteal phase when compared to the follicular phase. There appears to be a thermogenic effect of the increased progesterone levels during the menstrual cycle, causing an increase in basal metabolic rate (19). This is believed to be a result of the increased concentration of circulating progesterone during the phase. Therefore, changes in estrogen and progesterone concentrations during the menstrual cycle may influence thermoregulatory function in females during an exercise challenge. Although we did not directly measure thermoregulatory responses during the exercise intervention in the heat, estrogen's effects following eight and twelve days of therapy in the present experiment indicate that the elevated thermotolerance could be indicative of enhanced heat loss responses. Indeed, the reduction observed in resting, baseline core temperature suggests that estrogen could play a role in centrally lowering the set point about which body temperature is regulated, which in turn could exert its effects during exercise, blunting the rise in core temperature throughout the duration of the heat tolerance test. This would allow for an increase in heat loss responses at any given core temperature during the exercise intervention.

Body Fluid Composition. Plasma volume expansion is closely associated with estrogen therapy in both humans and rats (3, 21, 34). An increase in plasma volume corresponds with a reduction in hematocrit in estradiol-treated animals, with no alterations in erythrocyte volume (3). Previous reports in the literature indicate that estrogen is effective at lowering Hct measurements in resting animals (2,3). It is suggested that a lower Hct following estradiol administration is a strong indicator of plasma volume expansion (2). Plasma volume in both resting and exercise conditions is a major factor influencing thermoregulation. During exercise, interstitial fluid levels are reduced due to sweat formation and fluid shifts which tend to induce hypovolemia, compromising circulatory and thermal regulation (6). Therefore, hypervolemia, commonly associated with trained or heat acclimatized subjects, allows considerable loss of body water before plasma volume returns to pre-heat exposure or pretraining values (14). Consequently, it is reasonable to postulate that hypervolemia can improve performance by inducing better muscular perfusion, by limiting the reduction in cardiac output due to the decrease in plasma volume during exercise, and by facilitating thermoregulation by increasing skin blood flow (26). Plasma volume expansion observed as an adaptive response to acclimation to heat is associated with increased sweat rate, increased evaporative

heat loss, and decreased skin temperature during heat (6, 26). These thermoregulatory adaptations correlate highly with plasma volume and appear to play significant roles in enhancing heat tolerance by producing a decreased core temperature and reduced strain as acclimation occurs (6). By increasing the cutaneous perfusion, hypervolemia enhances the body's ability to regulate body temperature during exercise. Conversely, it has been demonstrated that the core temperature in dehydration is always found to be higher than in euhydration (7). The role of hypervolemia as a function of estradiol-induced plasma volume expansion as it relates to thermoregulatory responses has not been fully established. Marked reductions in Hct after estrogen therapy in ovariectomized rats is associated with an elevation in thermoregulatory responses, determined by evaporative water loss, while resting in the heat (2). Therefore, plasma volume expansion could play a beneficial role in heat loss mechanisms in the rat. Unfortunately, we did not directly measure baseline plasma volume or hematocrit values. However, we may speculate that estrogen therapy elevates plasma volume, reducing hematocrit, which in turn would increase evaporative cooling and enhance the animal's ability to perform exercise in the heat.

Metabolic Responses. The administration of estrogen may improve exercise performance in the heat by promoting liver and muscle glycogen sparing. It is well established that during prolonged exercise glycogen stored in liver and muscle tissue is utilized as a metabolic fuel source (8, 11, 13, 28). Depletion of muscle glycogen may play a significant role in the development of exhaustion during prolonged strenuous exercise (1, 10). Furthermore, when previously sedentary individuals engage in aerobic exercise training, the duration of time they can maintain a given submaximal work load is typically elevated (11). One mechanism whereby this may occur could involve a reduction in the rate of carbohydrate utilization by elevated oxidation of fatty acids. Early studies in the literature indicate that fatty acid oxidation inhibits carbohydrate utilization in isolated perfused rat heart tissue, limiting both glucose uptake and glycolysis (24, 25, 29). In addition, high concentrations of fatty acids has been demonstrated to slow carbohydrate utilization in the fast-twitch skeletal muscle in exercising rats (30). Further, the rate of muscle tissue uptake of plasma fatty acids is proportional to the concentration of fatty acids to which it is exposed (9, 16). When plasma free fatty acid levels were artificially elevated in rats prior to an exercise exposure, the animals ran for a significantly longer duration of time when compared to the controls, suggesting a marked influence on endurance perhaps due to enhanced muscle fatty acid oxidation (11). Therefore, an increase in the availability of fatty acids could be a significant factor in reducing glycogen depletion and enhancing endurance in trained compared to untrained individuals.

5. Summary. In conclusion, the evidence presented in this study suggests that administration of estradiol for eight and twelve days increases thermotolerance in ovariectomized rats. In addition, the data are indicative of a time course of hormonal therapy necessary to enhance performance during an exercise and heat challenge in ovariectomized rats, and that estrogen's effects on thermotolerance reach a plateau within eight days of treatment. These results could be due to enhanced thermoregulatory responses during exercise in a warm environment. Furthermore, estradiol-induced plasma volume expansion could also contribute to improving thermoregulation. Both of these factors would play a role in elevating evaporative cooling and heat loss mechanisms. In addition, estradiol treatment may result in a decreased rate of tissue glycogen utilization secondary to an estradiol-mediated increase in the availability of lipid substrate during the exercise intervention in the heat.

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Tables

Table 1. Effects of estradiol treatment on T_c, heating duration, heating rate, and heating rate as a function of mass in ovariectomized rats.

Group	Initial T_c, °C	ΔT_c, °C	Time to 40.4°C	Heating Rate, °C/min	Heating Rate/Mass, $°C \cdot kg^{-1} \cdot min^{-1}$
Vehicle					
4 Day	37.3±0.1	3.1±0.1	20.99±1.05	0.151±0.005	0.541±0.020
8 Day	37.3±0.1	3.1±0.1	20.30±1.19	0.155±0.007	0.550±0.020
12 Day	37.4±0.0	3.0±0.0	20.41±0.87	0.148±0.006	0.526±0.024
Estradiol					
4 day	37.4±0.1	3.0±0.1	17.66±1.53	0.175±0.008†	0.7078±0.034†
8 Day	36.8±0.2*†	3.6±0.2*†	44.77±8.93*†	0.091±0.011*†	0.383±0.042*†
12 Day	37.0±0.1*†	3.4±0.1*†	31.00±2.01*†	0.112±0.004*†	0.454±0.018*†

Values are means ± SE; n = 6 rats per group. T_c, colonic temperature. *Significantly different from 4 Day within a treatment group. †Significantly different from time-matched Vehicle. Differences were considered significant at the P<0.05 level.

Table 2. Effects of estradiol on body weight, hematocrit, and plasma osmolality of ovariectomized rats.

Group	Pre-Weight, g	Post-Weight, g	ΔWeight, g	Hct, %	Posm, mosmol/kgH_2O
Vehicle					
4 Day	273±2.2	269±3.5	3.7±0.3	38.1±0.01	336.7±1.36
8 Day	281±3.9*	276±3.9*	4.8±0.8	37.3±0.01	341.2±4.45
12 Day	283±5.6*	278±5.5*	4.3±0.8	36.5±0.01	334.2±1.67
Estradiol					
4 Day	248±3.0†	243±1.8†	5.3±1.7	35.8±0.01†	355.2±6.34†
8 Day	237±4.0*†	230±5.1*†	6.7±1.5	35.6±0.01†	346.0±3.15
12 Day	237±1.5*†	232±1.5†	4.0±0.4	35.5±0.01†	343.8±4.14

Values are means ± SE; n = 6 rats per group. Hct, hematocrit; P_{osm}, plasma osmolality. Note that P_{osm} was lower in estradiol- compared with vehicle-treated rats. *P<0.05 vs. 4-day treatment within a group. †P<0.05 vs. time-matched vehicle group.

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