Two years of combined high-intensity physical training and heat acclimatization affect lymphocyte and serum HSP70 in purebred military working dogs

Yaron Bruchim,1 Itamar Aroch,1 Ady Eliav,1 Atallah Abbas,2 Ilan Frank,4 Efrat Kelmer,1 Carolina Codner,1 Gilad Segev,1 Yoram Epstein,3 and Michal Horowitz2

1The Hebrew University Veterinary Teaching Hospital, Koret School of Veterinary Medicine, The Hebrew University of Jerusalem; 2Laboratory of Environmental Physiology, Hadassah Medical Center, The Hebrew University of Jerusalem; 3Heller Institute of Medical Research, Chaim Sheba Medical Center, Tel Hashomer, and Tel-Aviv University Medical School, Israel; and 4Israel Defense Force Military Working Dog Unit

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MILITARY WORKING DOGS ARE often trained and worked in rigorous and austere environments. In hot countries, these harsh conditions include prolonged high ambient temperatures during training, which may endanger the dogs and lead to the potentially lethal heatstroke syndrome (2, 6, 7, 11, 12). In a previous retrospective study in dogs we demonstrated that heatstroke was overrepresented in military working dogs during their first year of training compared with their presentation in the general hospital dog population (7). Hence, understanding the training conditions and the adaptive responses to environmental factors may enable provision of appropriate prevention and treatment. Unfortunately, there are few studies on the adaptive processes to adverse environmental conditions in military working dogs.

Previous studies from our laboratory show that adaptive responses to combined heat acclimation and exercise training are not simply additive, but are an outcome of tradeoffs or compromises to the effects of each stressor. Thus combined exposure to chronic heat and exercise training can be considered as a unique stressor (21). By and large, prolonged heat stress activates cellular mechanisms that combat thermal injury (20, 30), including cytosolic heat shock proteins (HSPs). The activation dynamics of these proteins contribute to determination of the threshold of heat-induced injury (27). Basal resting cellular HSP72 levels are low under comfortable conditions; however, HSPs are highly inducible under environmental and pathological stressors, and their roles include maintenance of cellular protein integrity by prevention of denaturation. HSPs also enhance recovery and confer thermal tolerance (17, 23, 39). This is achieved by the heat shock response (HSR). Originally, the term HSR referred to the phenomenon of cellular protection from severe acute heat stress following prior exposure to sublethal heat. However, this term now applies to other types of stressors such as oxidative stress, inflammation, and ischemic reperfusion injuries, with similar protective profiles (13, 14, 19, 20, 23, 30, 31, 37, 44). Furthermore, a variety of physiological situations result in a constitutive increase in HSP levels, including exercise and heat acclimation (30–33, 35).

In addition to its intracellular functions, HSP72 is secreted from healthy and necrotic cells (5, 24), exerting an extracellular function and acting as a danger signal as part of the immune response (3, 9, 32, 42). Studies in humans and pigs have shown that extracellular HSP72 (eHSP72) is a reliable marker of heat stress and heat acclimation (1, 33, 35, 36, 43). Another study demonstrated a significant increase in eHSP72 concentration during and immediately after physical exercise (43).

Studies of long-term adaptation of human populations transferred to geographical regions of high ambient temperature and exposed to combined heat and exercise demonstrated improvement in exercise performance and thermotolerance (4, 16). However, these studies primarily examined physiological parameters, and thus our knowledge of

Address for reprint requests and other correspondence: Y. Bruchim, Emergency and Critical Care Medicine Unit, The Hebrew Univ. Veterinary Teaching Hospital, Koret School of Veterinary Medicine, The Hebrew Univ. of Jerusalem, P.O. Box 12, Rehovot 76100, Israel (e-mail: bruchim@agri.huji.ac.il).
cellular adaptive markers in such subjects is very limited. Given the improved thermal tolerance and exercise performance, we hypothesize that concurrent cellular adaptive processes do occur.

Therefore, the aim of this study was to examine whether long-term, combined exercise endurance training and heat acclimatization to high environmental heat loads induces cellular adaptive processes. Exercise performance parameters and lymphocyte hsp72 mRNA and HSP72, as well as eHSP72 levels were used as molecular/protein markers of acclimatory responses. We hypothesized that with improvement in the physical performance and acclimation process, HSP basal levels and transcription would increase, as would their induction during the stress. Military working dogs undergoing seasonal training over a period of 2 yr and tested in the harsh summer period were used as a model. Given that cellular adaptive/protective mechanisms are evolutionarily conserved, we found that this model reflects adaptive processes in general.

MATERIALS AND METHODS

Animals, Physical Performance Test, and Sampling

Fifteen young, purebred, healthy, thermally naïve, untrained Belgian Malinois dogs imported from Western Europe to Israel by the Israeli Defense Force Military Working Dog Unit (IDFMWDU) were included in the study. The dogs were examined upon arrival in Israel, and monitored throughout their 2-yr training period. The training protocol of the IDFMWDU was individualized for each dog on the basis of progress and performance. Study dogs were gradually and progressively trained by increasing the time and distance of treadmill exercise by 10% every 2 wk during the study period. The training regimen included two to three bouts of treadmill exercise of between 2 and 8 km in an acclimatized room (22°C), outdoor exercise between 3 and 10 km, and 10–20 min following outdoor obstacle trails under conditions of high heat and humidity. The study was approved by the Israeli Defense Force ethical committee.

Three treadmill physical performance tests (PPTs) were conducted in an acclimatized room (22°C). The first was performed at the beginning of the training period, shortly after the dogs arrived in Israel (April 2011, early spring), the second was conducted 6 mo later (September 2011, end of summer), and the third one was conducted a year later (September 2012). All PPTs, including running distance, time, and running speed were determined individually on the basis of the training regimen of each dog. The military working dogs were held in ambient temperature kennels during resting hours. The PPTs were initiated at 8 A.M. Each dog handled by his guide was taken to the training acclimatized room (22°C) in which several physiological parameters were recorded, including heart rate (HR), rectal temperature (TREC) and respiratory rate. HR was measured by palpation of the femoral artery, whereas TREC was measured by fast-responding electrical thermometer as previously described (7). Thereafter, each dog performed its regular treadmill training. Immediately at the end of the training session, each dog’s physiological parameters were recorded (post-PPT).

Cephalic venous blood samples were collected in potassium-EDTA-containing tubes and in plain tubes with gel separators (AMSINO International, Pomona, CA) pre-PPT, immediately post-PPT, and 45 min post-PPT using a standard venipuncture technique. Samples in the plain tubes were allowed to clot and were centrifuged within 2 h of collection, the harvested sera were separated and stored at −80°C pending analysis. Blood samples in EDTA tubes were stored at 4°C pending analysis.

Definition of Environmental Heat Stress and Collection of Meteorological Data

All meteorological data [environmental temperature, humidity, and discomfort index (DI)] of the summer training period (2010–2011) were collected from the Israeli National Meteorological Service (INMS). The DI was calculated as \([\text{wet bulb globe (C°)} + \text{dry bulb globe (C°)}]/2\). This index combines air temperature, humidity, airflow, and radiant heat to measure heat stress disorders in humans. The DI is subdivided into mild (22.1–24), moderate (24.1–28), and severe (>28) on the basis of definitions used by the INMS.

Lymphocyte mRNA and Protein Extraction

Lymphocytes were concentrated using Ficoll (Ficoll-Paque PLUS; GE Healthcare, Sweden). Each blood sample was diluted 1:2 with PBS. The blood-PBS solution was then loaded into 10 ml of Ficoll reagent and centrifuged for 10 min (1,500 g at room temperature). The buffy coat was then removed and diluted with 15 ml of PBS. A series of cleansing centrifuges were conducted (2,500 g at room temperature). The clean pellet was homogenized in 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH) and stored at −80°C until analysis.

Lymphocyte mRNA. Quantitative real-time RT-PCR was used to measure hsp72 (qRT-PCR; ABI Prism 7000 Sequence Detection System, Applied Biosystems). Total lymphocyte RNA was extracted using TRI Reagent (Molecular Research Center) according to the manufacturer’s instructions (27). Twenty microliters of homogenate containing 10 μl of SYBR Green Master Mix (Applied Biosystems), 500 nM each of the forward and reverse primers, and 5 μl of diluted cDNA mixture was prepared. The appropriate cDNA dilution was determined using calibration curves established for each primer pair. The thermal profile for SYBR Green qRT-PCR was 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. To ensure equal amounts of initial mRNA, β-actin was used as a housekeeping gene.

Cellular protein fractionation. Total cytosolic and mitochondrial protein fractions were fractionated by electrophoresis on 9% polyacrylamide gels under denaturing conditions (Laemmli, UK) by cleavage of structural proteins during assembly of the head of bacteriophage T4, and later transferred onto nitrocellulose membranes, and then blocked for 1 h in PBS containing 5% dried skimmed milk powder. The membrane was then probed overnight at 4°C with the primary antibody (polyclonal anti-HSP72; Stressgen, Victoria, BC, Canada) at 1:1,000 dilution. Repeated washings were performed following a 1-h incubation period at room temperature with horseradish peroxidase-conjugated polyclonal anti-rabbit IgG (Stressgen). β-Actin was used as a housekeeping protein. Specific antibody binding was detected using enhanced chemiluminescence (Beit Haemek Biological Industries, Beit Haemek, Israel) and visualized by exposure of an X-ray film to the membrane. Band densities were calculated using TINA software (Raytest, Straubenhardt, Germany) as previously described (28, 30, 40, 41).

Serum HSP70 Analysis

A commercially available enzyme immunoassay kit was used according to the manufacturer’s instructions (Assay Designs, New York, NY). Briefly, each sample was divided into three aliquots, and analyses were carried out in triplicate. Samples were placed into wells containing monoclonal antibodies for HSP70, and incubated for 2 h at room temperature. The wells were then washed with a Tris-HCl-buffered solution (washing buffer), leaving only plate-bound HSP70. Polyclonal antibodies against HSP70 (Biolegend) were added and incubated for 1 h (room temperature), then washed and stained with a horseradish peroxide-catalyzed reaction for 30 min. The dyed solution was read by a spectrophotometer (i-MARK microplate reader; Bio-Rad, Hercules, CA) at 450 nm. The amount of signal read by the
spectrophotometer was directly proportional to the sample HSP70 concentration. The average optical density of each triplicate was calculated and used for statistical analysis.

Statistical Analysis

The Kolmogorov-Smirnov test was used to assess distribution of the continuous parameters. Continuous parameters available at only two points and that were normally distributed (e.g., HR, temperature) were compared before and after exercise using the paired *t*-test. Changes in continuous parameters (e.g., lymphocyte mRNA, serum protein) available at three different time points (pre-PPT, post-PPT, and 45 min post-PPT) was performed using the Friedman test (because some data were not normally distributed). The Wilcoxon signed rank test was used to compare individual pairs (e.g., pre-PPT vs. post-PPT). All tests were two-tailed, and *P* ≤ 0.05 was considered significant. Data are presented as median and range. Statistical analyses were performed using a statistical software package (SPSS 17.0 for Windows; SPSS, Chicago, IL).

RESULTS

Signalment, and Pre-PPT, Immediately Post-PPT, and 45-Min Post-PPT Vital Signs

This study included 15 purebred Belgian Malinois IDFM-WDU dogs (8 males and 7 females) with a mean age of 1.94 yr (±0.6) and mean body wt of 27.7 kg (±3.6) at the start of the study (Table 1). Most of the dogs were panting before and after the PPTs, except 6 dogs before the first PPT with a mean respiratory rate of 27 breaths/min (±5.7), 1 dog before the second PPT with 32 breaths/min, and 2 dogs before the third PPT with 20 and 32 breaths/min.

The mean exertional treadmill distance in the first, second, and third PPTs were 2.68 km (±0.69), 4.82 km (±2.18), and 6.36 km (±1.93), respectively (Table 1). The second and third PPTs were significantly longer than the first (*P* ≤ 0.05 and *P* ≤ 0.05, respectively).

The mean ΔT° in the first PPT was significantly higher (mean 1.68 ± 0.24°C; *P* = 0.001) than the second (mean 0.89 ± 0.18°C) and third PPT (mean 1.12 ± 0.21°C) (Fig. 1, left). Similarly, the increase in HR after the first PPT was significantly greater than HR recorded after the second and third PPTs (*P* = 0.009, and *P* = 0.025, respectively) (Fig. 1, right). The monthly average temperature, humidity, and DI were similar with no significant difference during the 2 yr of the study (Table 2).

Lymphocyte HSP72 Protein Levels (HSP72)

The median lymphocyte HSP72 to β-actin ratio in the study dogs pre-PPT, immediately post-PPT, and 45 min after the first PPT were 0.47 (range 0.23–0.86), 0.57 (range 0.33–0.92), and 0.64 (range 0.45–0.86), respectively (Fig. 2). The median post-PPT lymphocyte HSP72 to β-actin ratios were significantly higher in all three PPTs compared with the basal pre-PPT ratio (*P* = 0.05, *P* = 0.016, and *P* = 0.0001, respectively). However, there were no significant increases in the basal level and induction of HSP72 immediately post-PPT and 45 min post-PPT in the three consecutive PPTs (*P* = 0.65, *P* = 1.00) (Fig. 2).

Lymphocyte hsp72 mRNA

In the first PPT the median lymphocyte hsp72 to β-actin mRNA ratio pre-PPT was significantly (*P* = 0.007) lower than immediately post-PPT and 45 min post-PPT (1.00, 4.22, and 12.82, respectively). Similar results were observed in the second and third PPTs; however, the increases in mRNAs immediately post-PPT and 45 min post-PPT were progressively higher in the second and third PPTs compared with those in the first PPT (*P* < 0.0001 for both) (Fig. 3).

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**Table 1. Physical examination data and running distance of 15 military dogs in training in three consecutive PPTs during the study**

<table>
<thead>
<tr>
<th>PPT</th>
<th>Age, yr mean (SE)</th>
<th>Body weight, kg mean (SE)</th>
<th>Distance, km mean (SE)</th>
<th>Time, min mean (SE)</th>
<th>Speed, HR, bpm mean (SE)</th>
<th>ΔHR, bpm mean (SE)</th>
<th>Pre-PPT T°, °C mean (SE)</th>
<th>ΔT°, °C mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 2011</td>
<td>2.00 (±0.15)</td>
<td>27.77 (±0.99)</td>
<td>2.68 (±0.19)</td>
<td>21.75 (±1.55)</td>
<td>7.2 (±0.3)</td>
<td>96.9 (±3.9)</td>
<td>46.4 (±6.2)</td>
<td>38.8 (±10.1)</td>
</tr>
<tr>
<td>September 2011</td>
<td>2.25 (±0.15)</td>
<td>28.08 (±0.87)</td>
<td>4.82 (±0.66)</td>
<td>28.12 (±5.52)</td>
<td>8.4 (±0.3)</td>
<td>87.7 (±4.7)</td>
<td>20.9 (±5.4)</td>
<td>38.71 (±0.11)</td>
</tr>
<tr>
<td>September 2012</td>
<td>2.75 (±0.15)</td>
<td>28.78 (±1.19)</td>
<td>6.36 (±0.54)</td>
<td>32.54 (±3.60)</td>
<td>8.8 (±0.4)</td>
<td>83.1 (±4.4)</td>
<td>38.1 (±6.3)</td>
<td>38.37 (±0.07)</td>
</tr>
</tbody>
</table>

bpm, beats per minute; HR, heart rate; PPT, physical performance test; T°, rectal temperature.

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*Significant increase in heart rate in the first year, *P* = 0.009.
Lymphocyte hsp72 mRNA to Lymphocyte HSP72 Ratio

The ratio between median mRNA/protein in lymphocytes increased significantly during the study period when comparing untrained naïve dogs to acclimatized trained dogs. This significant difference was observed before, immediately after, and 45 min after PPT (Fig. 4).

Serum eHSP70 Profile

In the first PPT, median eHSP70 level, presented as average optical density in the study dogs before, immediately post-PPT, and 45 min post-PPT were 0.13 (range 0.12–0.31), 0.15 (range 0.11–0.45), and 0.16 (range 0.14–0.40), respectively. Basal levels of eHSP70 in the first PPT were significantly (P = 0.002) lower than corresponding values in the second and third PPTs (Fig. 5). Additionally, the increase in extracellular HSP (eHSP) level after the second and third PPTs was significantly (P = 0.002) greater than after the first PPT (Fig. 5). Figure 6 presents the eHSP70/ΔT\text{re} relationship at the end of the PPT for each individual dog. It is clearly observed that the ratio increases markedly in dogs that underwent training for a year or two, irrespective of changes in ΔT\text{re} (P < 0.001). For comparison, a similar analysis is presented for lymphocyte mRNA (Fig. 6, right). This analysis shows that the highest induction of hsp72 mRNA occurred in the last year of training. ΔT\text{re} had no influence on eHSP70 levels, whereas the cumulative training had a clear effect.

DISCUSSION

In this study, serum and lymphocyte HSP72 protein and transcript levels were measured in dogs subjected to combined exercise training and heat acclimatization over a 2-yr period. The training protocol resulted in a profound enhancement of aerobic power and physical performance over the study period, reflected by the lower rise in post-PPT, T\text{re}, and HR in the face of significantly longer treadmill running distances. The study shows that hsp72 mRNA induction progressively increased throughout the entire study period, and during each PPT, peaking at 45 min post-PPT. Concomitantly, the induction of lymphocyte protein HSP72 was stable. Considering these facts, it seems that the cellular/molecular adaptive tools to maintain HSP72 homeostasis are enhanced under these conditions. There was also a significant rise in basal extracellular HSP following acclimatization and training.

In the present study, military working dogs were used as a model of the superimposition of endurance exercise on seasonal acclimatization to high ambient temperatures and heat stress, as occurs in the summer in hot regions. In some studies on heat acclimation, basal T\text{re} was significantly lower after a 7-day acclimation period, and this was considered to be indicative of acclimation (8, 34, 35). In contrast, in our study median basal T\text{re} was stable following a long summer period that included combined heat stress and exercise training. This discrepancy between studies may be due to the excitement of

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**Table 2. Monthly meteorological data during the summers of 2011–2012 of the training period**

<table>
<thead>
<tr>
<th>Month</th>
<th>Maximum Temperature*</th>
<th>Average Temperature*</th>
<th>Wet Temperature*</th>
<th>Humidity*</th>
<th>DI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>May</td>
<td>27.7</td>
<td>28.6</td>
<td>22.4</td>
<td>22.7</td>
<td>17.8</td>
</tr>
<tr>
<td>June</td>
<td>29.5</td>
<td>30.6</td>
<td>25.3</td>
<td>25.3</td>
<td>21.0</td>
</tr>
<tr>
<td>July</td>
<td>32.3</td>
<td>33.3</td>
<td>26.6</td>
<td>28.9</td>
<td>23.5</td>
</tr>
<tr>
<td>August</td>
<td>32.4</td>
<td>33.5</td>
<td>28.0</td>
<td>29.2</td>
<td>23.6</td>
</tr>
<tr>
<td>September</td>
<td>31.3</td>
<td>31.8</td>
<td>26.7</td>
<td>27.2</td>
<td>22.5</td>
</tr>
</tbody>
</table>


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**Fig. 2.** Top: lymphocyte HSP72 to β-actin protein ratio of dogs in the study under basal conditions (pre-PPT), at the end of the PPT (post-PPT), and 45 min post-PPT. Each box represents the median and range. *Significant increase in lymphocyte HSP72 immediately post-PPT in all PPTs of the study. Bottom: protein bands of individual dogs during the three PPTs (lanes a–g).
the dogs before the PPT, because measurement was performed in the treadmill room, not in the kennels, but it may also reflect changes between short- and long-term acclimation. In rodents, for example, 1 mo of acclimation does not change basal body temperature (30). Previous acclimation studies have shown increased stroke volume, cardiac output, and plasma volume. These parameters were not measured in the present study; however, the enhanced physical performance supports an increased cardiovascular capacity. The fact that HR remained unchanged between the second and third PPT despite increased endurance (duration, less Tre elevation) suggests that cardiovascular fitness improved in this period. In contrast to humans, a change in respiratory rate during physical activity is a good indicator of fitness or physical ability in dogs.

We suggest that the continuous intensive training regimen that our dogs underwent throughout winter and spring induced adaptation to exercise training. Thus the results of the second and third PPTs, conducted at the end of the summer when ambient temperatures peak, specifically reflect the impact of heat acclimation superimposed on exercise training. Similar interpretations have been made in studies of heat acclimation of human subjects (22, 25, 26, 33).

Acclimation and acclimatization to heat stress decrease the temperature threshold at which heat dissipation mechanisms are activated (18). Additionally, the machinery of the HSR, which is predisposed to acute heat stress, provides sustained cytoprotection and an earlier enhanced HSR (18, 30). Exercise training under conditions of heat stress in humans induces similar responses (1, 33, 35). In dogs acclimatized to outdoor climate when seasonal changes in heat balance were evaluated, Tre stability was suggested (38). In the present study, the dogs preserved their HSP72 homeostatic profile over the 2-yr follow-up period; however, significant changes occurred in the transcriptional response over time. In the trained and acclimated dogs, post-PPT hsp72 mRNA levels in the second and third PPTs were significantly higher than were corresponding levels in the first PPT. The greater hsp72 mRNA elevation observed between the second and third PPTs is inversely correlated with the lower ΔTre post-PPT compared with the naïve condition, indicating that combined heat acclimation and physical training involves augmentation of mRNA production (18). The more pronounced increase in HSP transcript in the face of a lower Tre increase may suggest that training and acclimatization affect the temperature threshold for induction of HSR and, in turn, leads to delayed heat injuries in the acclimated groups.

The significant increase in the ratio between hsp72 mRNA and HSP72 emphasizes that higher hsp72 mRNA levels are needed to produce the same HSP72 protein levels in trained dogs. Notably, heat-acclimated rats under sedentary conditions showed a similar phenomenon (30), suggesting perhaps that acclimation, either to heat only or to heat and exercise, induces a similar response. In the current study, due to ethical limitations, only two post-PPT samples were taken. Only additional delayed blood sampling will indicate whether sensitivity change in translational system occurs when acclimation and exercise training are combined. This issue was beyond the scope of the current study.

In acclimated trained dogs, basal eHSP72 levels were higher compared with levels in naïve dogs. In addition, there was a significantly higher increase in eHSP72 post-PPT in the acclimated dogs, with no changes in this increase between the second and third PPTs. This increase was not related to changes in Tre, nor to the training period (PPT2 vs. PPT3; Fig. 6), suggesting that once an acclimated HSP72 mRNA/HSP72 protein exercise was achieved, the dominant determinant of their elevation was the cumulative effect of exercise. Fig. 6, right, suggests that the transcriptional machinery of the lymphocytes was similarly affected. The endogenous cellular signaling leading to this effect is yet unknown. Given that ΔTre is ruled out, we speculate that hormonal or metabolic reflex
changes upon acclimation and training may play a role (15, 29, 35). The increase in eHSP in acclimated dogs is in agreement with previous findings (10, 33, 35). Recently, it was reported that initial training induced an increase in eHSP levels that diminished the need for further induction of the protein during exercise (33). However, the follow-up period in our study was much longer than the above-mentioned study (2 yr vs. 15 days). Furthermore, in the previous study, the PPTs were conducted daily, whereas in the present study, the dogs did not undergo PPTs within the days preceding the final PPT.

It has been suggested that the rate of eHSP induction may be used as an easily accessible biomarker of stress (in contrast to its cellular transcript) (15, 35). The current study shows that in the acclimated phenotype, it is the magnitude of rise of eHSP—namely, peak/basal ratio during the exercise bout—that may serve as a good indicator of acclimation and physical performance in military working dogs, and potentially in populations at risk. It may also be useful for screening for the risk of thermal tolerance and heatstroke in dogs under harsh training and environmental conditions.

In the present study, the increase in eHSP72 level immediately post-PPT was followed by its decline at 45 min post-PPT. This pattern of a gradual decrease in serum HSP72 level differs from the dynamics observed in cellular protein HSP72 and its mRNA levels, which further increased post-PPT. We have no clear explanation for this discrepancy and may only speculate that cellular permeability is affected by the different physiological conditions.

One limitation of this study is that the effects of acclimation to environmental heat and physical performance could not be assessed individually. However, on the basis of studies of human elite trainees who underwent acclimation (25), we are confident that our second and third PPTs, conducted at the end of the summer, reflect the effects of acclimatization to heat superimposed on endurance training on the HSP profile. Additionally, because of the differences in exercise endurance time due to ethics limitations, blood sampling for HSP analyses at the end of the PPT differed due to the individual difference in exercising time (Table 1). Nevertheless, 45 min post-PPT sampling was equal for all sessions. Hence, differences detected for these samples reflect real differences throughout the elapsed years. In the current study, additional blood samples to test for an HSP72 delayed response were not allowed by ethics limitations.

Additionally, our current HSP profile corresponds to lymphocytes only. Other cells were not studied.

In conclusion, this is the first long-term study to evaluate the dynamics of serum and cellular HSP72 protein levels and their expression in lymphocytes, and their association with physical endurance and performance, as well as with acclimation to heat stress and training in working military dogs. The techniques we employed can be used for future studies. Such studies should be on a larger scale and should investigate and characterize the dynamics and role of cytosolic and eHSP70 in response to heat acclimatization and its relation to physical endurance in working dogs.

**GRANTS**

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