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Detection of Inducible HSP70 as a Measure of Heat Stress in Mammalian Tissue

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Detection of Inducible HSP70 as a Measure of Heat Stress in Mammalian Tissue

Submitted in partial fulfillment of the requirements for graduation with honors from the
Department of Natural Sciences at Carroll College, Helena, MT

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Abstract

The North American Pika, *Ochotona princeps*, is a keystone high altitude species whose populations have been declining and whose distributions have been receding to higher elevations in the Great Basin. The present study proposes that these population shifts may be occurring because *O. princeps* are thermally stressed, as indicated by high levels of the inducible form of Heat Shock Protein 70 (HSP70). *Ochotona princeps* blood samples were collected from a range of elevations at different temperatures. Western Blot analysis was used as a qualitative measure to detect the presence of HSP70 in various mammalian and cell line samples to prepare for examination of *O. princeps* samples. Thermally treated Human Embryonic Kidney (HEK293) showed a higher expression of HSP70 than room temperature HEK293 cells. Western Blot Analysis of various mammalian tissues revealed interference in visualization of blood plasma HSP that may be attributed to similar sized protein hindrance from proteins such as albumin. Though this study does not provide tangible results on HSP70 levels across different elevations for *O. princeps*, it does offer useful mechanisms for studying proteins as indicators of thermal stress in mammals.

Introduction

The North American pika, *Ochotona princeps*, is a keystone species of high altitude ecosystems that is highly susceptible to thermal stress (Lawlor, 1998). In the last 100 years, nearly a third of *O. princeps* populations in the Great Basin area have disappeared (Beever et al., 2003). Accompanying this disappearance has been a general population shift to higher altitude regions of their respective mountain ranges (Grayson, 2005). There are three main hypotheses that attribute these biogeographic alterations to climate change and warmer temperatures (Mead et al., 1982). The first hypothesis cites the decrease in insulating snow depth as the cause of the pika recession. With lower annual snow depths, the *O. princeps* populations could be vulnerable to the extremely cold winter temperatures of their environment (Beever et al., 2003). Second, Mead et al. (1982) suggest that warming trends in high altitude portions of the Great Basin have reduced *O. princeps* habitat sufficiently to affect their food supply and force the populations' retreat to higher altitudes. Third, Beever et al., (2003) state that the shift in *O. princeps* distribution may be due to direct thermal stress. A more specific study that analyzes the effect of heat directly on *O. princeps* physiology to determine if the species is thermally stressed has yet to be done. By quantifying thermal stress in *O. princeps*, we can better understand why the populations of this important species are declining and why their distribution is contracting.

Although there are many indirect indicators of thermal stress, such as free-radical oxidation, the best way to directly measure thermal stress is by quantifying the up regulation of the inducible form of Heat Shock Protein 70 (HSP70) (Morimoto, 1998). Two forms of HSP70 exist in mammals: a constitutively expressed form known as HSPA8 and an inducible form called HSP70 (Feder and Hofmann, 1999). The constitutive form is produced at a constant level in vertebrates as a chaperone protein that facilitates the translocation of proteins into the endoplasmic reticulum (Feder and Hofmann, 1999). In contrast, the concentration of the inducible form fluctuates within the organism according to environmental factors such as high ambient temperatures (Morimoto 1998). The up regulation of HSP70 during heat stress

minimizes protein degradation caused by such stress by aiding in the folding and structural maintenance of cellular proteins (Morimoto 1998). Therefore, the increased production of the inducible form of HSP70 within an organism can be used as an indicator of thermal stress (Morimoto 1993). Western blot Analysis, a common immunoblotting assay, is one of the most well-known and effective techniques for both qualitative and quantitative protein analysis (Phizicky and Fields, 1995).

Western blot analysis has previously been used to study HSP70 from tissue samples, such as muscle and liver, extracted from mammals (Gong et al., 2012). *O. princeps*, however, has recently been considered for the U.S. Fish and Wildlife Service's Endangered Species list (Dept. of Interior, 2010). Therefore, the invasive tissue removal required for these techniques must be avoided. Furthermore, Hunter-Lavin et al. (2004) have shown that HSP70 is sufficiently present in blood serum derived from a simple blood draw and the blood plasma fractionation technique (Brodiewicz-Proba, 1991). In conjunction with the Hunter-Lavin et al. (2004) study that reveals the presence of HSP70 in human blood serum, Hatting et al. (1983) show that the blood serum protein composition of mammals is conserved. Unfortunately, there is no literature on Western blot analysis of proteins from mammalian whole blood or plasma. Therefore, in the present study, significant technique development was required to prepare and analyze blood samples for the immunoblotting. This includes utilizing a protein sample preparation technique before making plasma specific adjustments to the Western blot procedure.

In the present study, I proposed the use of Western blot analysis on *O. princeps* plasma to determine if there is a significant difference in HSP70 levels between populations at low altitudes and populations at high altitudes, based on the temperature differences associated with this contrast in altitudes. A climate study by Giorgi et al. (1997) shows that geographic areas at lower elevations generally have higher average temperatures than areas at higher elevations. I hypothesize that *O. princeps* are being subjected to direct thermal stress as quantified by higher levels of HSP70 in lower altitude populations due to their exposure to this higher mean

temperature. If samples taken from high altitude sites early in the summer have lower expression of HSP70 than lower altitude sites taken later in the summer, as quantified by Western blot analysis, then my hypothesis will be supported.

Materials and Methods

In order to locate *O. princeps* populations from the desired elevations, local *O. princeps* data on populations and elevations was utilized from a Global Information Systems (GIS) file from April Craighead of the Craighead Institute in Bozeman, Montana. By navigating this database with Carroll College's GIS software, we selected four populations with elevations that were consistent with the objectives of this experiment. These altitudes satisfied the present study's aims of gathering data at both low and high elevation sites, which were determined to be about 1980 meters and 2740 meters, respectively. In addition, samples were collected from these sites during June and again in August, which constituted the aforementioned early and late sampling periods.

Ochotona princeps were initially trapped by setting out a combination of 61 cm long Havahart 1088 Collapsible Animal Cage Traps and 25 cm Sherman Folding Vole Traps. Due to the mammal's affinity for small crevice locomotion, traps were placed tightly next to rocks on level surfaces amongst the talus. The traps were then covered with flat rocks and foliage to provide a deceptive, crevice-like appearance. Apples were used as bait and fruit was rubbed inside the trap and on the rocks adjacent to it. To determine if a trap had been occupied, we remained nearby and returned to check the traps every few hours. At some sites, such as the Deer Lodge site and the Red Lodge site, overnight trappings were done in addition to the day trappings. Dr. Brandon Sheafor (pers. comm.) was the advisor for proper trapping protocols.

The sample collection began with the removal of the *O. princeps* from the cage with a strong mesh bag encasing one end while the cage was inverted. Once the *O. princeps* was in the bag, it was anesthetized with cotton ball slightly saturated with isoflurane, a common veterinary anesthetic provided to us by Matt Blanford of Apex Veterinary Clinic (Parasuraman et al., 2010).

Anesthesia was administered only until there was a slight reduction in respiratory rate. After the anesthetic was in effect, a toenail was clipped from one of the hind limbs to generate a small blood flow. If this was inadequate, as it sometimes was, the feet were palpated to stimulate blood flow. Blood was collected using 20G needles on 1 mL syringes that were large enough to not cleave the red blood cell membranes, which would contaminate the plasma with red blood cell intracellular proteins. 50 – 200 uL of blood was collected from each animal. Upon collection, the blood was centrifuged immediately with a field centrifuge and plasma was separated from the sample. After blood sampling, flour was applied to the nail to clot the wound and blood and plasma samples on dry ice to preserve them until they could be returned to the -80 °C freezer, where they were stored until further investigation.

The analysis of *O. princeps* plasma in the laboratory consisted of utilizing Western blot analysis for protein identification and quantification. Before using *O. princeps* samples, however, Human Embryonic Kidney (HEK 293) cells and blood samples from rats were used to test our protein purification and qualification. Dr. Stephanie Otto-Hitt generously donated the HEK293 cell techniques for this study.

For the HEK293 cell line, the heat shock protocol suggested by Nijhuis et al., (2008) was used. These heat shocked HEK293 cells were then mixed with sample buffer, which consisted of a 19:1 ratio of Lameli Loading buffer to Beta Mercaptoethanol (BME). Both heat shocked and room temperature HEK293/sample buffer samples were analyzed by Western blotting to detect an up-regulation in HSP70. For later analysis of *O. princeps* samples, the heat shocked HEK293 cells served as a positive control while untreated HEK293 cells served as a negative control.

Along with the HEK293 cells, blood from rats was analyzed with Western blotting to ensure the techniques reproducibility and accuracy for *O. princeps* blood. Furthermore, blood from both a heat shocked rat and a normal rat was used for a preliminary insight into distinguishing various levels of HSP expression in blood samples. To develop the heat-treated rat, a chamber was immersed in a water bath with a heater. The rat's internal temperature was

raised from 32°C to 36°C, as indicated by rectal temperature using the Field Metabolic System (FMS) by Sable Systems International. After 24 hours, the approximate time for full HSP70 expression after heat shock, the rat was lethally exposed to CO₂ (Feder and Hofmann, 1999). Dr. Sheafor provided blood extraction techniques (pers. comm.). Blood was taken directly from the right ventricle and stored at -80°C before being analyzed by Western Blotting. The samples were centrifuged at 13000 rpm for three minutes at room temperature in order to extract plasma to analyze alongside muscle and liver samples for comparison of HSP70 concentrations.

The Western Blot gels were made according to standard sodium dodecyl sulfate (SDS) gel crafting procedures and as described by Dr. Otto-Hitt (pers. comm.). The stacking and running portions of the gel were poured with 7.5% and 10% acrylamide, respectively. The gel was loaded in a Bio-Rad Mini Trans-Blot Cell (Cat # 153 BR 77315) and filled with a 1X SDS buffer made by diluting Bio Rad's 10x Tris/Glycine/SDS Buffer (Cat. #161-0732) with DI water. Before loading samples into the gel, they were mixed and placed in boiling water for 5 minutes to ensure protein denaturation for proper protein separation. The gels were run at 90 volts for 20 minutes followed by 120 Volts for 40 minutes after running by using the Bio-Rad PowerPack Basic power supply (Cat #151-0544). The gel was then transferred to a nitrocellulose membrane from Bio-Rad (Cat. # 162-0215) that was pre-soaked in a 1X Tris/Glycine Transfer buffer with methanol. This buffer consisted of 800 mL of DI H₂O, 100 mL of methanol, and 100 mL of 10x Tris/Glycine Buffer that was made by mixing 72 g glycine and 15 g tris base in 500 mL DI H₂O. The gel was transferred at 100 V for an hour.

After the transfer, the membrane was soaked in 1X Tris-Buffered Saline (TBS), for ten minutes on a mixer plate. Fifty mL of 1% blocking solution was added to 1 g of nonfat dry milk and allowed to stir for 30 minutes in 100 mL of Tween Tris-Buffered Saline (TTBS). After this initial blocking, 6 uL of blocking/antibody solution (Enzo Hsp70/Hsp72 mAb ; Cat. # ADI-SPA-810) was added along with 50 mL of new blocking solution and was left to stir overnight. The

following day, the membrane was subjected to 3 ten-minute washes in TTBS. Then, 6 mL of blocking solution was mixed with 2 mL of GAM-HRP secondary antibody from the Bio-Rad Immuno-Blot Assay Kit (Cat. #170-6463-1) and added to the membrane for an hour. This was followed by two ten-minute washes in TTBS and a final wash in TBS. Color development was achieved by the procedure provided with the Immuno-Blot Assay Kit.

Results

There was a total of seven *O. princeps* plasma samples acquired. Early season populations at high altitudes were inaccessible due to large amounts of snow. **Table 1** describes the populations we sampled.

Table 1. *Ochotona princeps* sampling classifications and locations.

Pika #	Classification	Date	Site	Elevation
1	Early Low Elevation	6/27/13	Lincoln	~1980 m
2	Early Low Elevation	7/1/13	Lincoln	~1980 m
3	Early High Elevation	7/5/13	Red Lodge	~2845 m
4	Early Low Elevation	7/9/13	Deer Lodge	~2070 m
5	Late High Elevation	7/26/13	Elkhorn	~2835 m
6	Late High Elevation	8/14/13	Red Lodge	~2845 m
7	Late High Elevation	8/16/13	Red Lodge	~2845 m

The results of preliminary western blot analysis on rat tissue are shown in **Figure 1**. The strong band for the positive control, the recombinant protein in lane two, is demonstrative of effective primary and secondary antibody binding. Lanes four and five demonstrate unexpected non-specific antibody binding in rat plasma.

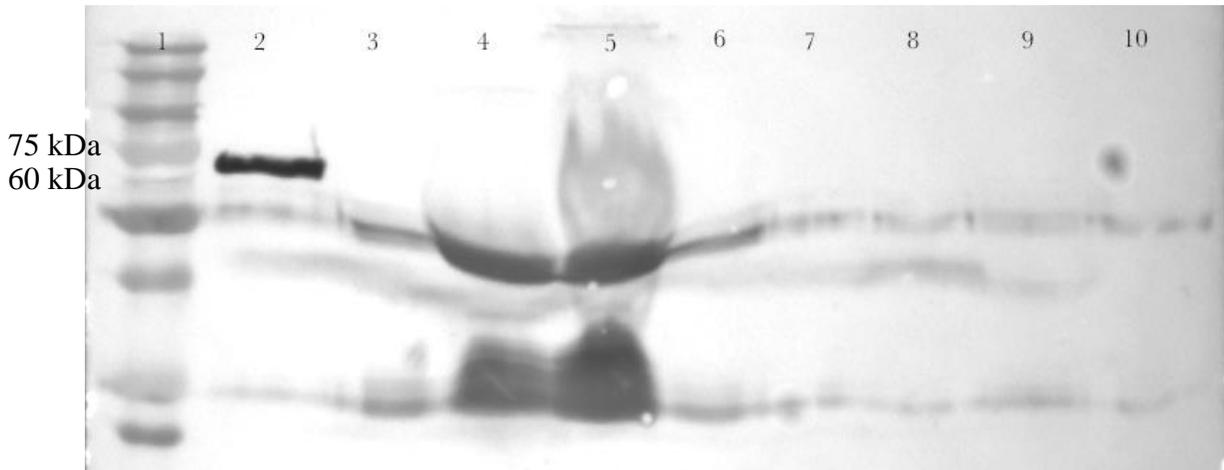


Figure 1.
Western blot analysis of HSP70 in rat tissue using mAb-HSP70/72a.
 1) Ladder 2) Recombinant HSP70 3) Empty 4) Diluted rat plasma (5 uL plasma) 5) Concentrated rat plasma (10 uL plasma) 6) Empty 7) Muscle
 8) Liver 9) Empty 10) Empty

To investigate the odd banding pattern observed in rat plasma in **Figure 1**, the experiment was repeated by adding 1uL of HSP70 recombinant protein to each sample with the addition of the heat-treated and normal HEK293 cell line samples as additional controls. Neither of the HEK293 cell line controls were spiked with recombinant HSP70. **Figure 2** shows the results of this analysis. The strong band in lane two is a diluted recombinant HSP70 that is aligned approximately at 70 kDa between the 60 and 75 kDa markers on the ladder. Lane three shows the successful expression of HSP70 in the HEK293 cells that were heat-treated. Lane four's faint band is reflective of the absence of the inducible form of HSP70 in room temperature HEK293. The recombinant HSP70 shows up in spiked muscle and liver samples, but is masked in the plasma samples.

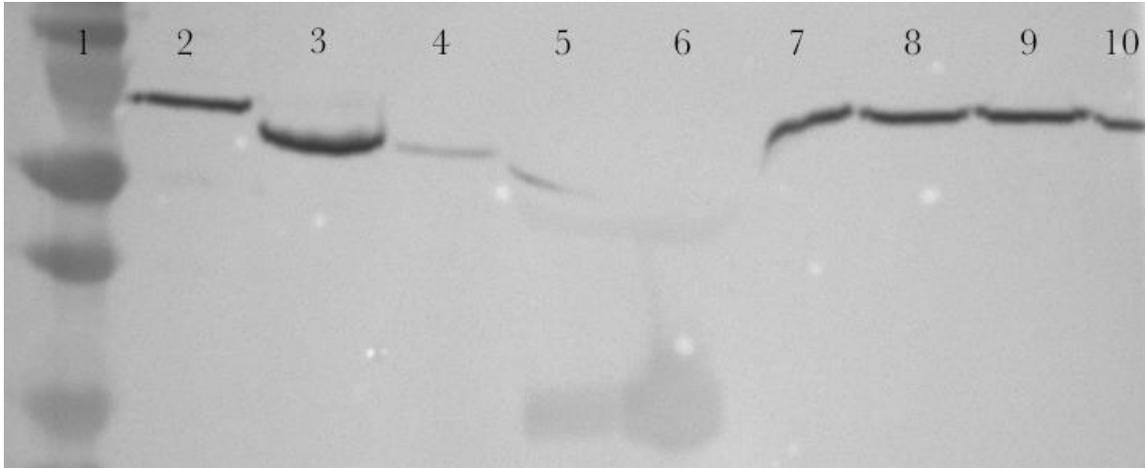


Figure 2.

Western blot analysis of HSP70 in HSP spiked rat tissue and HEK293 cells using mAb-HSP70/72a. 1) Ladder 2) Recombinant HSP70 3) Heat-shocked HEK 293 4) Room temperature HEK 293 5) Diluted plasma spiked with 1uL HSP70 recombinant 6) Concentrated plasma spiked with 1 uL HSP70 recombinant 7) Diluted muscle spiked with HSP70 recombinant 8) Concentrated muscle spiked with HSP70 recombinant 9) Diluted liver spiked with HSP70 recombinant 10) Concentrated liver spiked with HSP70 recombinant.

Discussion

As of this writing, no western blot analysis data for *O. princeps* has been obtained. The Western blot analysis of rat tissue was repeatedly unsuccessful and prevented analysis of *O. princeps* tissue. Therefore the initial hypothesis of increased HSP70 expression in early season, lower elevation populations cannot be rejected nor confirmed. However, the preliminary western blot analyses on HEK293 cells and rat tissue samples have resulted in significant progress towards developing a technique to analyze *O. princeps* samples.

The western blot in **Figure 2** highlights several key aspects of this progress. First of all, the smeared banding pattern exhibited in lanes five and six is demonstrative of sample interference with detection of HSP70. Even though the plasma, liver, and muscle samples in lanes five through ten were all spiked with one uL of HSP70, the plasma samples did not show the definite HSP70 bands like the other samples. This indicates that there may be a protein in the

blood sample that is interfering with either the running portion or the antibody attachment portion of the western blot protocol. This is problematic because only samples of whole blood and plasma can be obtained from *O. princeps*. Therefore, a way to visualize HSP70 expression in these samples must be developed.

One of the strongest options for properly visualizing the HSP70 in blood or plasma samples is the removal of the hindering protein. The protein that is likely responsible for this interference is albumin, which has a weight of 67 kDa and is the most abundant protein in mammalian blood. Albumin is similarly sized to HSP70 and may be competing with HSP70 for the binding of the primary and secondary antibodies (He and Carter, 1992). To remove albumin and purify the samples so that HSP70 can be more effectively quantified, a two-step Co-Immunoprecipitation (Co-IP) technique should be used.

The Co-IP technique isolates HSP70 from other proteins in samples prior to analysis by Western blotting. This technique features the attachment of protein specific antibodies to sepharose beads, which are mixed with the sample to bind the target protein (Phizicky and Fields, 1995). The beads are then washed and the target protein is isolated for Western blotting (Phizicky and Fields, 1995). The Co-IP insures that the Western blot samples are free of proteins that may cause deficiencies in HSP70 antibody binding (He and Carter, 1992).

The first Co-IP will remove albumin and the second Co-IP will isolate HSP70. I hypothesize that the results of this Co-IP experiment will clearly show a difference in HSP70 expression in heat-treated rats samples relative to normal rats samples and can be later applied to *O. princeps* samples.

The faint band in lane five near 60 kDa provides another useful result of the banding pattern seen in the plasma samples in lanes five and six of **Figure 2**. The presence of this band at a lower plasma concentration than the adjacent, more concentrated plasma lane, may imply an inverse correlation between density of plasma in the sample and the ability to detect HSP70. This relationship is consistent with my hypothesis that a protein in the plasma sample is interfering

with our ability to accurately detect HSP70 expression. A western blot with a gradation of plasma samples, all spiked with one uL of HSP70, would be an effective method to test this hypothesis. If the band strength of HSP70 decreases with an increase of plasma concentration, then there is a substance in the plasma sample that is masking HSP70 expression.

One of the other possible solutions for better determining the presence of HSP70 in plasma samples may be to utilize a higher percentage poly-acrylamide gel. This would enable greater protein separation, allowing the 3 kDa difference between Albumin and HSP70 to be more distinct.

Another significant result seen in **Figure 2** is the successful detection of an up regulation of the inducible form of HSP70 in heat-treated HEK293 cells. The strong band for the heat shocked HEK293 cells in lane three is a significant contrast from the dim band for the room temperature HEK293 cells in lane four. This demonstrates the ability to detect a difference in the expression of HSP70 between a heat shocked species and a normal, room temperature species. This is an extremely promising step in detecting varying levels of HSP70 expression in the different *O. princeps* populations from different elevations and times of year. In conjunction with the double Co-IP procedure, a western blot could be used to distinguish between varying concentrations of HSP70 in *O. princeps* samples as was originally planned.

The final interesting result of the analysis of the HEK293 cells is the faint band in lane four of **Figure 2**. This may be due to spillover from lane three. More likely, however, the band may be a result of a low level of HSP70 expression due to normal fluctuations in temperature that would be significant enough stimulate the expression of HSP70 during the sampling process. These temperature fluctuations may elicit expression of a small amount of the inducible form of HSP70, which can be visualized in the faint band at around 70 kDa.

Despite the promising results for the utilization of Western blot analysis for detection of HSP70 expression in *O. princeps* blood tissue, a recently developed product from Enzo (Cat. # ADI-EKS-700B) may hold the answer for the most efficient and accurate quantification of

HSP70 levels. This immunoassay is specifically designed for HSP70 detection in blood plasma and serum. It has been validated in its detection of HSP70 in heat-stressed samples and may be a possible alternative to my suggested purification-intensive protocol.

Even though there is plenty more to be done in order to effectively determine the expression of HSP70 in *O. princeps*, the present study offers several key preliminary results on which future research should be based. By implementing the double Co-IP technique and possibly the ELISA immunoassay, future research can demonstrate whether or not HSP70 quantification is an effective way of measuring heat stress on this important species. Indeed, it is crucial that we continue study of this organism and explore the possible explanations for its changing distribution. Research on the North American pika, with its diminishing high altitude existence, may be providing a crucial insight into the future of other high altitude species and high altitude ecosystems as a whole.

References

- Beever, E. A., Brussard, P. F., Berger, J. 2003. Patterns of apparent extirpation among isolated populations of pikas (*Ochotona princeps*) in the Great Basin. *Journal of Mammalogy*. 84:37-54.
- Brodniewicz-Proba, T. 1991. Human Plasma Fractionation and the Impact of New Technologies on the Use and Quality of Plasma-derived Products. *Blood Reviews*. 5: 245-257.
- Dept. of Interior. 2010. Endangered and Threatened Wildlife and Plants; 12-month Finding on a Petition to List the American Pika as Threatened or Endangered. *Federal Register*. 75: 6437-6471.
- Feder, M.E and G.E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61: 243-282.
- Giorgi, F., Hurrell, J.W., Marinucci, M.R. 1997. Elevation Dependency of the Surface Climate Change Signal: A Model Study. *Journal of Climate*. 10: 288-296.
- Grayson, D.K. 2005. A brief History of Great Basin Pikas. *Journal of Biogeography*. 32(12): 2103-2111.
- Gong, T.W., Fairfield, D.A., Fullarton, L., Dolan, D.F., Altschuster, R.A., Kohrman, D.C., Lomax, M.I. 2012. Induction of Heat Shock Proteins by Hyperthermia and Noise Overstimulation in *Hsf1*^{-/-} Rats. *JARO*. 13: 29-37.
- Hatting, J., Bomzon, L., Marcus, E., Jooste, C., Ganhao, M.F., Cheney, C.S., de Vos, V. 1983. Concentration and composition of plasma proteins in wild mammals. *Comp. Biochem. Physiol.* 75(3): 441-445.
- He, X.M. and D.C. Carter. 1992. Atomic structure and chemistry of human serum albumin. *Nature*. 358: 209-215.

- Hunter-Lavin, C., Davies, E.L., Bacelar, M.M., Marshall, M.J., Andrew, S.M., Williams, J.H. 2004. Hsp70 release from peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun.* 324(2): 511-517.
- Lawlor, T.E. 1998. Biogeography of Great Basin mammals: paradigm lost? *Journal of Mammalogy.* 79: 1111–1130.
- Mead, J.I., Thompson, J.I., Van Devender, T.R. 1982. Late Wisconsinan and Holocene fauna from Smith Creek Canyon, Snake Range, Nevada. *Transactions of the San Diego Society of Natural History.* 20: 1–26.
- Morimoto, R.I. 1993. Cells in stress: Transcriptional Activation of Heat Shock genes. *Science.* 259: 1409-1410.
- Morimoto, R.I. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* 12: 3788-3796.
- Nilhuis, E.H., Poot, A.A., Feijen, J., Vermes, I. 2008. HSP70 and p53 responses after heat treatment and/or x-irradiation mediate the susceptibility of hematopoietic cells to undergo apoptosis. *Int. J. Radiat. Biol.* 84(2): 99-105.
- Parasuraman, S., Raveendran, R., Kesavan, R. 2010. Blood sample collection in small laboratory animals. *J. Pharmacol. Pharmacother.* 1(2): 87–93.
- Phizicky, E.M. and S. Fields. 1995. Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* 59: 94–123.