**Changes in Concentrations of Heat Shock Protein 60, 70 and 90 of a Wild Songbird in Responses to Distinct Stress Challenges**

by

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## Abstract

Wild songbirds, such as the House Finch (*Carpodacus mexicanus*) require physiological mechanisms to maintain the homeostasis in face of stress threats. One of the primary mechanisms to protect system integrity is production of heat shock proteins (HSPs). Heat shock proteins belong to chaperone families that play key roles in supervising the folding structures of proteins, helping vertebrates to suppress and degrade denatured proteins in multiple stressful environments. HSP90, HSP70 and HSP60 are three major HSP families that have been used as biomarkers of stress in a wide range of vertebrates. In my thesis, I used heat shock protein concentrations of male House Finches to evaluate the responses of wild songbirds under multiple stressful environments: thermal stress, disturbance stress and pathogen stresses. My study is the first study to compare different HSPs responses of wild songbird under sequential and distinct stressful environments.

HSP concentrations from blood samples of birds were measured by western blot assay and were adjusted by internal controls to counteract variations in western blotting efficiency. Male house finches were trapped a feeding stations in Arizona in hot and cool weather. Birds were then transported to aviary and kept in cages to experience sequential stresses.

In chapter one, we evaluated the HSPs responses of wild male house finches in response to thermal stress. Through comparisons of the HSP concentrations in response to hot versus cool weather condition, we found that both HSP70 and HSP60 showed significantly increased concentrations in hot weather, while concentration of HSP90 did not change. The results

demonstrated that HSP70 and HSP60 were sensitive in response to thermal stress and HSP70 can be a reliable indicator of thermal stress of male house finches in wild.

I chapter two, male house finches were transported to Auburn, AL from Arizona by van. We then measured the HSP responses of birds under sequential and distinct stressful environments: hot weather, transportation, recovery period, and MG (*Mycoplasma gallisepticum*) pathogen infection. Results showed that all HSPs—HSP90, HSP70 and HSP60—had significantly different responses of changes in concentrations across those the four conditions. Although the responses of the three heat shock proteins varied, concentrations of all three HSPs were highest in MG-infection condition. MG infection was demonstrated to have the most significant effect on HSP responses. In addition, due to different concentration pattern of each HSP, studies of different stressors should choose different HSPs.

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# Chapter One
# Effects of Heat Stress on Circulating Heat Shock Proteins in a Wild Songbird


## Abstract

Heat shock proteins (HSP) are chaperone molecules that play a critical role in protecting the integrity of proteins. In vertebrates, HSP90, HSP70 and HSP60 have been found to respond to a variety of stressors but few studies have been conducted with animals in natural environments. We measured the response of HSP90, HSP70 and HSP60 in the blood of a wild songbird, the house finch (*Carpodacus mexicanus*) captured in under hot weather conditions versus cool weather condition. We observed that the three HSP had different responses to thermal stress. Levels of HSP90 remained constant between hot and cool conditions; HSP70 was present in modest concentrations in cool weather and rose to high concentration in hot weather; HSP60 was barely detectable in cool weather but rose to high concentration in hot weather. These observations suggest that HSP60 and HSP70 can be used as indicators of heat stress in songbirds. Whether these HSP can be used as indicators of a broader range of stressors remains to be determined.


## Introduction

To maintain vital life function it is imperative that organisms preserve the integrity of their proteins. A variety of environmental factors including heat, toxins, oxidative stress, pH imbalance, and disease caused by pathogens can impede the correct folding of newly created

proteins (Beckmann et al. 1992, Hagiwara and Nagata 2012, Doyle et al. 2011, Roberts et al. 2010) or disturb the structure of already folded proteins (Beckmann et al. 1992, Deane and Norman 2010, Wang et al. 2010). Because of these threats to protein integrity, organisms from bacteria to human produce chaperone molecules that help to maintain the integrity of proteins across a range of environmental perturbations.

Heat shock proteins (HSPs) are perhaps the most ubiquitous and abundant cellular chaperones constituting about 20% of the cellular proteins of animals following exposure to stressful conditions (Bhardwaj et al. 2012). HSPs were originally identified in *Drosophila* when it was found that flies were better able to survive high temperatures if they had previous been exposed to a high temperature stress (Ritossa 1962, Rotossa 1996). It has since been discovered that production of various HSPs is induced by not just heat but by a variety of stressors including ethanol (Beck 1995), heavy metals (Lindquist 1986), and pollutants (Feder and Hofmann 1999).

Three major classes of HSPs─HSP90, HSP70 and HSP60─have been identified in vertebrates, including birds, fish and mammals (Salway et al. 2011). HSP90 usually works as a complex that incorporates other chaperones and co-chaperones (Kosmaoglou et al. 2008) to escort the proteins to tertiary structures (Malthews 1992). In various species of vertebrates, increased levels of HSP90 have been shown to be associated with the tolerance of hypothermia, cell proliferation, and cell cycle control (Herring and Gawlik 2007). HSP90 was the only HSP that was differently expressed in the spleen of House Finches (*Carpodacus mexicanus*) after infection by *Mycoplasma Gallisepticum* (Bonneaud et al. 2011).

HSP70 is the central chaperone family that co-operate with other proteins like HSP90 or CHIP (Hsc70-interacting protein; Hsc70 is the isoform of HSP70) to regulate protein folding and to maintain of the structure of proteins in both cytosol and mitochondrial matrix (Kriegenburg et

al. 2012). In some vertebrates, HSP70 has been observed to be more sensitive to environmental perturbations than HSP90 or HSP60, so HPS70 has been proposed to be a particularly good biomarker of stress (Dehbi et al. 2010, S. Lewis et al. 2000). In study of lymph node of laboratory mice, HSP70─but not HSP90 or HSP60─was induced by exposure to the toxic mercury chloride (Albers et al. 1996), and HSP70 but not HSP60 was induced by the immune system activation in male pied flycatchers (*Ficedula hypoleuca*) (Sanz et al. 2004). HSP70 also shows increased expression in the hippocampus of laboratory mice, and in the hearts and lungs of chicken following exposure to cold (Filipovic et al. 2005, Hernandes et al. 2002). HSP70 is also related to tolerance for hypothermia of broiler chicken (Liew et al. 2003).

HSP60 belongs to the classic chaperone families that forms a ring-crystal shape and mainly assists in protein folding (Kosmaoglou et al. 2008). HSP60 is thought to function primarily in the tolerance of hypothermia (Herring and Gawlik 2007), but also it plays a key role in the assembly of proteins within cells and in the immune system of vertebrates (Young 1990). In several studies no change in HSP60 was observed in response to heat or cold stress while in the same studies HSP70 showed significantly elevated concentrations in laboratory mice, human and juvenile Hamadryas baboons (*Papio Hamadryas*) (Albers et al. 1996, Kim et al. 2001, Dehbi et al. 2010)

HSPs can be detected in most organs and tissues of vertebrates including blood. Circulating HSPs in blood had been shown to be related to heat stress in vertebrates like domestic broiler chicken (Zulkifli et al. 2003a), wild turkey (*Meleagris gallopavo*) (Wang and Edens 1993), domestic goats (Meza-Herrera et al. 2005), juvenile Hamadryas baboons (*Papio hamadryas*) (Dehbi et al. 2010), and Common Carp (*Cyprinus carpio*) (Lund et al. 2003, Ferencz et al. 2012). Because they are nucleated, the red blood cells of birds can synthesize HSP and production of HSP in blood enables birds to mobilize HSP to sites in the body where it is needed (Feidantsis et

al. 2010, Feidantsis et al. 2012). Moreover, HSP70 in blood was found to remain elevated after stressful temperatures were halted while HSP70 in other organs rapidly return to baseline levels (Lund et al. 2003).

Despite a growing interest in HSP as potential indicators of exposure to stress in wild vertebrates (Clark and Peck 2009, Ryan and Hightower 1996), little is known about how different HSPs respond to temperature stress in wild vertebrates. In this study, we assessed the quantities of circulating HSP90, HSP70 and HSP60 in wild House Finches captured during a period of very high temperatures and during a period of moderate temperatures. Our goal was to assess the utility of using some or all these heat shock proteins as indicators of exposure to thermal stress in songbirds.

## Materials and Methods

### *Field sampling*

Our goal was to characterize heat shock protein levels in House Finches during some of the hottest days of the summer in Arizona (AZ). These high summer temperatures have been confirmed to trigger stress responses in AZ birds (Wolf and McKechnie 2012). In addition, we then collected finches at the same localities during mild weather when ambient temperatures approximated thermal neutrality (Salt 1952). On 11, 12, and 13 August 2010 two teams of researchers captured male house finches at baited feeding stations in Tempe and Green Valley, AZ, approximately 135 miles apart; hereafter we will refer to birds collected in August as "hot-weather birds". Thirty additional male house finches were captured in Tempe, AZ on 10, 17, 18 and 29 November 2011; hereafter we will refer to this group as "cool-weather birds". In August, the daytime high temperature on all three days of collection was 41.7$^{o}$C in Green Valley and 37.1,

39.6, 40.1$^{o}$C in Tempe (http://www.ncdc.noaa.gov/cdo-web/search). During the cool collecting period, daytime high temperatures were 21.6, 26.1, 25.0 and 23.2 $^{o}$C.

Within 1 hr of capture, a blood sample was taken from each bird and stored on ice. All blood samples were spun at a speed at 13000 rpm for 5 min at the end of each day and the packed red blood cells stored on liquid nitrogen in the field and in a -80 $^{o}$C freezer in the lab.

*Western Blot*

We performed a Western Blot assay to quality the concentrations of blood proteins following the protocol (presented in Tomas et al. 2004). After cells were broken by sonication, we standardized the protein concentrations in each sample.

Due to the protein difference, we designed to test HSP90, HSP70, and HSP60 on two gels. One gel was run to test HSP90, HSP60 and internal control; another one was ran to test HSP70 and internal control. We cut membranes to separate HSP90, HSP70, and HSP60. Mouse monoclonal antibody from Sigma Company was used to target and bind HSP90, other two mouse monoclonal antibodies from Abcam Company were used to target and bind HSP70 and HSP60. The antibodies were applied to each protein with following concentrations: anti-HSP90 1:1333, anti-HSP70 1:4000 and anti-HSP60 1:1333. Secondary anti-mouse antibody from Sigma Company was used to bind the primary antibodies with dilution 1:5000 to all proteins.

X-ray film images of proteins were scanned to create photographic images. Images were analyzed by UN-SCAN-IT version 8.1, gel version digitizing software to quantity the value of total pixel for the HSPs and internal control in each blot.

*Statistic analysis*

Each relative HSP concentrations of birds in hot and cool weather were used to do the data analysis with two-sample t-test in SAS software. We reported results for independent HSP (HSP90, HSP70, and HSP60). Means and standard deviations are given.

**Results**

We first compared the levels of circulating HSP in hot-weather birds captured in Tempe and in Green Valley. We found no significant differences in the levels of any heat shock protein between birds collected from these two sites in AZ (Figure 1, HSP90: t=-1.78, P=0.07; HSP70: t=-1.69, P=0.095; HSP60: t=-1.58, P=0.12; two-tailed t-test), so we pooled data from birds from the two locations for comparisons to cool-weather males.

For HSP90, we found no significant differences in circulating HSP between hot- than in cool-weather birds (Figure 2, t=-0.64, P=0.52). For HSP70, we found significantly higher concentrations of circulating HSP in hot- than in cool-weather birds (Figure 3, t=-7.27, P<0.0001). We measured low levels of HSP60 circulating in cool weather and higher levels during hot weather, and concentration of HSP60 was significantly higher in hot- than cool-weather conditions (Figure 4, t=-5.4, P<0.0001). Relative levels of all heat shock proteins in both hot and cool collecting conditions are summarized in Table 1.

**Discussion**

The thermal-neutral zone of house finches is between 24 and 32 $^{o}$C (Salt 1952). The daytime high temperatures during sample collection in November were between 21 $^{o}$C and 25 $^{o}$C, close to the thermal-neutral zone of the House Finches and in a range that should not induce thermal stress in active birds. Thus we used the samples taken in cool weather as a baseline from which to examine how HSP change in response to high temperatures. Our results showed that in

the cool-weather environment, both HSP90 and HSP70 circulated at modest levels, but that HSP60 was barely detectable. Daytime highs during sample collection in August were up to 10 $^{o}$C above thermal neutrality. In hot weather, the amount of circulating HSP70 and HSP60 significantly increased, while amount of circulating HSP90 did not change.

Our observation of no change in HSP90 in response to high temperature is inconsistent with observations of HSP90 responses in other taxas. In spleen tissue of laboratory mice (Albers et al. 1995), the adrenal gland tissue of American alligator (*Alligator mississippiensis*) (Kohno et al. 2010), the spleen of fish (*Cyprinus carpio*) (Ferencz et al. 2012), and the heart tissue of cold anoxic turtle (*Trachemys scripta*) (Stecyk et al. 2011) heat stress led to elevated HSP90 concentration. Prior to this study, the responses of HSP90 to heat stress had been reported to increase in two other species of birds (Wang and Edens 1993, Lei et al. 2009). The difference between our study and these previous studies may lie in the acclimatization of wild birds to summer temperatures in Arizona. Although it was very hot in Arizona during August collection and the birds seemed to be thermally stressed as indicated by panting, the condition under which we captured birds may not have been sufficiently thermally stressful to trigger an increase in HSP90. House Finches in southern Arizona are adapted in hot summer temperature. These birds may use mechanisms other than HSP90−including HSP70 and HSP60−to deal with the challenge to protein structure imposed by high but normal ambient temperature.

Consistent with previous studies, HSP70 showed an increase in response to high ambient temperatures. Heat stress has been showed to trigger an increase in HSP70 in virtually all the vertebrates studied to date, including laboratory mice (Beck et al.1995, Albers et al. 1996), domestic goats (Meza-Herrera et al. 2005), humans (Hayashi et al. 1991, Kim et al. 2001), juvenile Hamadryas baboons (Dehbi et al. 2010), common carp (Ferencz et al. 2012) and

domestic birds such as domestic chickens (Taylor et al. 2010, Zulkifli et al. 2003a, Givisiez et al. 1999, Hernandes et al.2002) and domestic turkey (Wang and Edens 1993). The birds in these previous studies were subjected to heat stress by elevating the temperatures in lab environments over a short time period. Our study is the first to show the responses of HSP70 of birds under chronic heat stress in the wild. Our observations support the idea that HSP70 can be a reliable, sensitive biomarker of thermal stress in wild vertebrates (Dehbi et al. 2010, Lewis et al. 2000). Whether HSP70 is a good indicator of a broader range of stressors in wild songbirds remains to be demonstrated.

Previous studies of HSP60 have not demonstrated a consistent response by this protein to heat stress among different vertebrates. For example, in the livers of laboratory mice (Schamhart et al. 1992) and in the embryonic tissue of zebrafish (*Danio rerio*) (Hallare et al. 2005), HSP60 rose in responses to high temperatures. However, in the thymi and spleens of laboratory mice (Alberts et al. 1996), HK-2 cells in human (Kim et al. 2001) and blood of Hamadryas baboons (Dehbi et al. 2010), HSP60 was not significantly affected by high temperature. Another study of sea bream (*Sparus (=Rhabdosargus) sarba)* has also found that hepatic HSP60 concentration stayed unchanged even after pathogen (*Vibrio alginolyticus*) infection (Deane and Woo 2005). We observed that unlike HSP70 and HSP90, HSP60 circulated at very low levels in non-stressful weather and then rose significantly during months with hot weather. These observations suggest that there may be a higher threshold for the production of HSP60 compared to HSP70.

Heat shock proteins are significant chaperone families that are involved in multiple cellular pathways in vertebrates, including the heat stress responses. In our study, we highlighted the HSPs responses to heat stress by observing male House Finches in hot versus cool weather condition. Ours is the first study to show the responses of HSPs of songbirds under chronic stress

in wild. We demonstrated that both HSP70 and HSP60, but not HSP90 can be induced by heat stress in wild songbirds. Understanding the range of stressors that affect HSPs will require further research.

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**Table 1,** Mean HSPs concentrations (±SD) of male House Finches in hot versus cool weather. Tempe and Green Valley are listed to compare the differences of HSPs levels between two sites.

| | Hot | | *Hot | Cool | p-value |
|---|---|---|---|---|---|
| | Tempe | Green Valley | | | |
| HSP90 | 1.03±0.79 | 0.74±0.68 | 0.90±0.75 | 1.04±1.24 | 0.52 |
| HSP70 | 2.95±1.43 | 2.42±1.12 | 2.74±1.35 | 0.64±0.72 | <0.0001 |
| HSP60 | 0.43±0.28 | 0.33±0.28 | 0.38±0.29 | 0.04±0.17 | <0.0001 |

p-value: difference of HSPs levels in hot versus cool weather ($p<0.05$)

*Hot: the mean HSPs concentrations in Tempe and Green Valley

**Figure 1** Comparison of the expression levels of heat shock proteins in the blood of House Finches captured in Tempe and Green Valley (GV), Arizona in hot weather. Relative intensities of HSPs are based on the total number of pixel per blot adjusted by concentrations of internal control to counteract variations in western blotting efficiency. Box-whiskers were used to provide the quartile values. The vertical line within each box was used to indicate the mean.

**Figure 1**

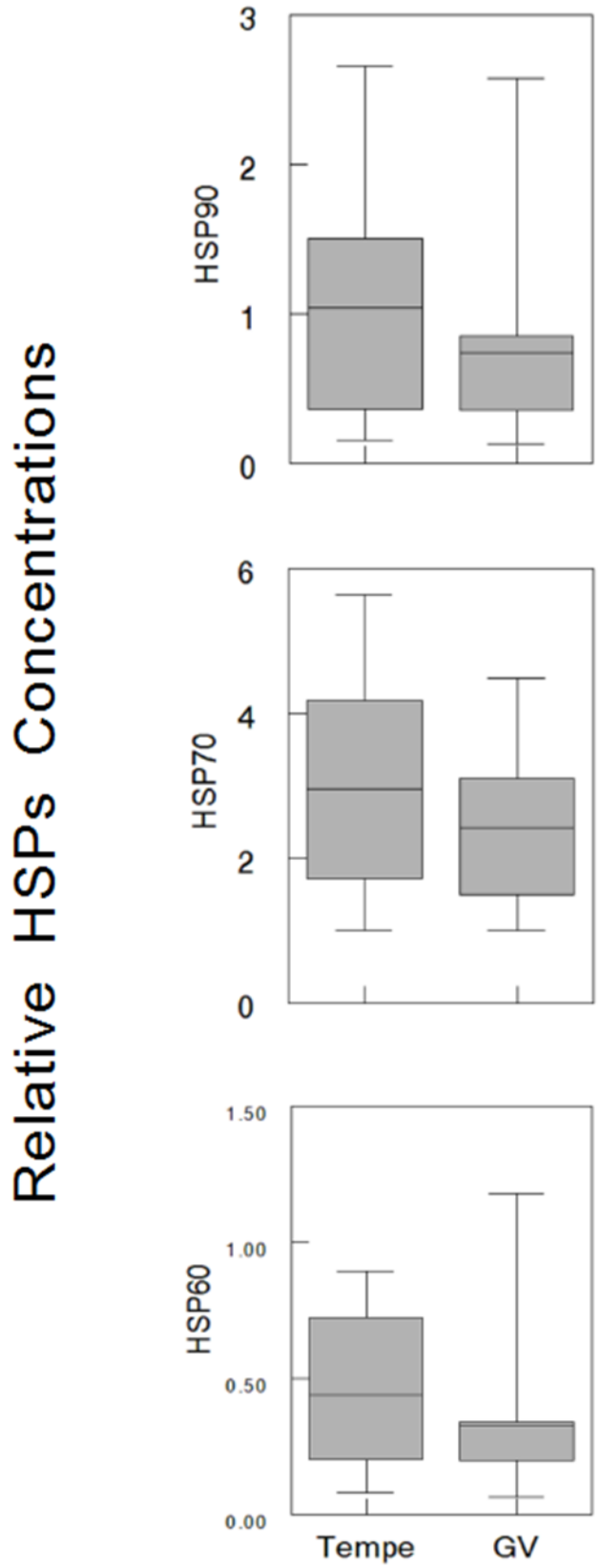
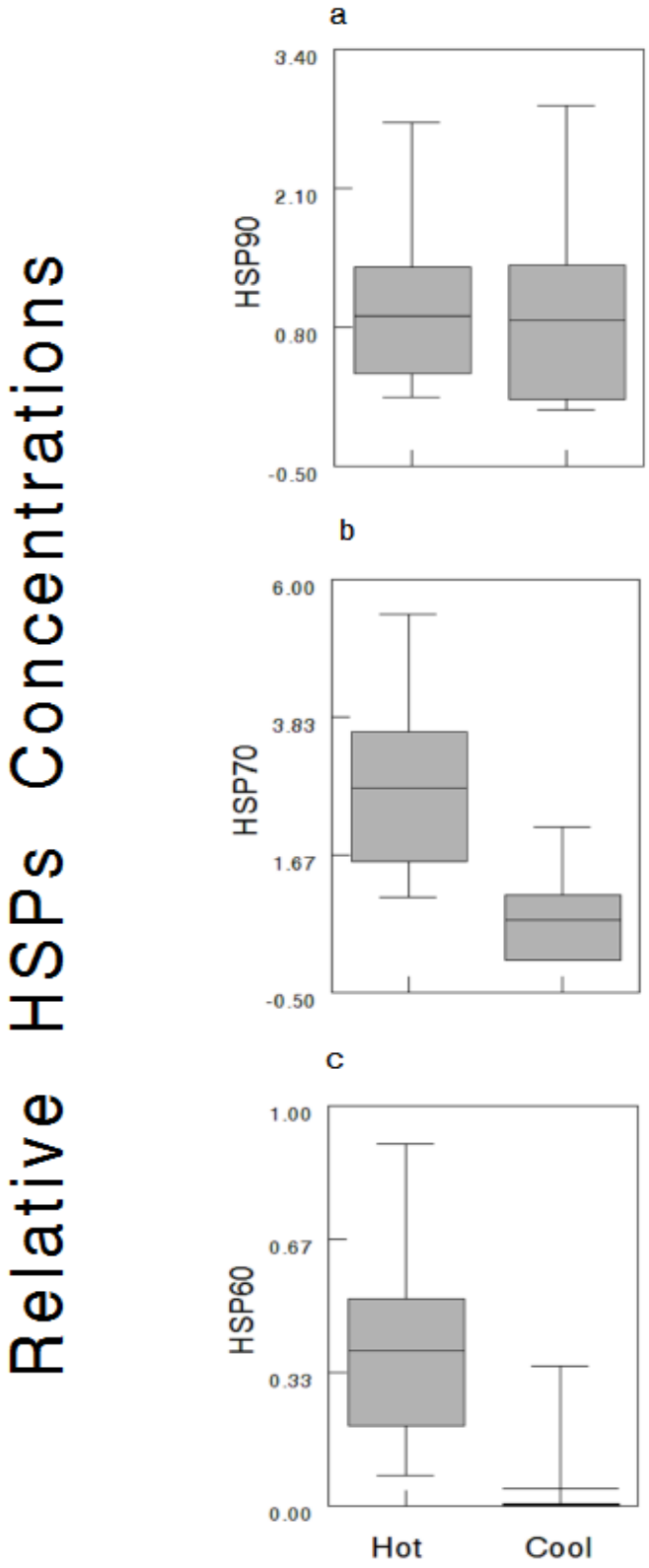

Relative HSPs Concentrations
HSP90
3
2
1
0
HSP70
6
4
2
0
HSP60
1.50
1.00
0.50
0.00
Tempe
GV

**Figure 2** Relative concentrations of HSP90 (a), HSP70 (b) and HSP60 in the blood of House Finches captured in hot weather versus cold weather. Relative intensities of HSPs are based on the total number of pixel per blot adjusted by concentrations of internal control to counteract variations in western blotting efficiency. Box-whiskers were used to provide the quartile values. The vertical line withinin each box was used to indicate the mean.

**Figure 2**


Relative HSPs Concentrations
a
3.40
2.10
0.80
-0.50
HSP90
b
6.00
3.83
1.67
-0.50
HSP70
c
1.00
0.67
0.33
0.00
HSP60
Hot
Cool

## Chapter Two
## Changes in the Concentrations of Heat Shock Protein 60, 70 and 90 in a Wild Songbird in Responses to Distinct Stress Challenges


### Abstract

We assessed the responses of male House Finches (*Carpodacus mexicanus*) to high ambient temperature, transportation in a vehicle, and infection by *Mycoplasma Gallisepticum* (MG) by measuring concentrations of HSP90, HSP70 and HSP60 in blood. We also monitored HSP levels during a 6-week period between transport stress and pathogen exposure. HSP90, HSP70 and HSP60 each showed significantly different expression across the four treatments, and among three environmental stresses. MG-infection was demonstrated to induce the highest concentrations of all three HSPs. These observations suggest that responses of each HSP were not consistent in the four distinct environments. We conclude that different patterns of three HSPs are probably based on their corresponding different functions.


### Introduction

In studies of how vertebrates respond to environmental stressors, physiological ecologists typically focus on endocrine or the immune function. Other stress-response system, in particularly heat shock proteins (HSPs) remains relatively little-studied in wild animals. Changes in concentration of HSPs could be an effective indicator of physiological characteristic of stressful environments in wild animals.

Heat shock proteins protect the integrity of proteins (Kriegenburg et al. 2012, Kosmaoglou et al. 2008). They supervise the newly formed proteins (Malthews 1992), and they protect the tertiary structure of already folded proteins (Beckmann et al. 1992, Deane and Norman 2010, Wang et al. 2010). Thus, HSP provide a critical function in the maintenance of homeostasis in cells. They increase in abundance in response to any environmental inducers that can denature proteins (Aufricht 2011). It has been shown that HSPs contents will increase to up to 20% of cellular proteins when animals were exposed to stresses (Bhardwaj et al. 2012). However, not all HSP will necessarily respond in the same manner to all stressors.

HSP families are named by molecular weight, and the three most abundant families are HSP60, HSP70, and HSP90. Although those three major HSPs share common chaperone functions, the concentration responses of different HSP in vertebrates are variable even were stimulated by the same environmental stress (Fu, chapter 1). HSP90, HSP70 and HSP60 sometimes cooperate to each other or other HSP families, fulfilling the chaperone functions (Ellis 1987) by supervising protein quality in cytoplasm or nucleus (Salfity and Knowlton 1996). HSP70 functions primarily in detecting denatured protein and degrading them to unfolded status. HSP60, in contrast, functions primarily in targeting unfolded protein and helping them to reach tertiary structure efficiently (Csermely and Yahara 2002). HSP90 is primarily involved in signaling transduction pathway, acting as hormone receptors (Pałyga and Kozłowski 2008). A study of spleen tissues of laboratory mice showed that HSP90 and HSP70 increase in response to thermal stress but HSP60 did not (Albert et al. 1996). Other studies on vertebrates also demonstrate the different responses of different HSPs to different stressors. In pied flycatchers (*Ficedula hypoleuca*), hatching induces HSP70 but not HSP60 (Sanz et al. 2004). In humans,

HSP70 but HSP60 showed increased concentrations in response to both heat and chemical ($CdCl_2$) stressors (Kim et al. 2001).

Little is known about how different HSPs repond to different types of stressors in wild songbirds. Most studies have compared the concentration responses of two heat shock protein families in only one stressful environment. In House Finches (*Carpodacus mexicanus*) we found that hot weather significantly increased concentrations of HSP70 and HSP60 but not HSP90 (Fu, Chapter 1). Disturbance stress is used to describe a series stress factors that trigger a "fight or flight" response by the sympathetic nervous system (Edward and Rykiel 1985). Disturbance stresses that have been studied previously in birds include human contact (Lynn et al. 2010), capture (Dickens et al. 2009), captivity, and transportation stress (Mitchell et al. 1992, Yalcin et al. 2004, Pijarska et al. 2006). In previous studies transportation stress has been of a few hours in duration. Transportation stress has been shown to induce negative effects on birds (Neves da Silva et al. 2007) and has been used to measure the survivability of birds by their coping abilities to this stress (Cheng and Jefferson 2008).

After being invaded by pathogens, intracellular and extracellular environments of cells of host vertebrates will be altered; hence the stress responses will be stimulated (Murray and Young 1992, Polla et al. 1995). HSP concentration responses in host have been demonstrated to be involved in post-pathogen infection in many vertebrates (Sterwat and Young 2004), including birds. In a study of the blood of blue tit (*Cyanistes caeruleus*), birds had higher HSP concentrations after parasite (*Haemoproteus spp*) infection (Martinez-de la Puente et al. 2011). High concentrations of HSP60 and HSP70 were associated with high immunity in three Antarctic penguin species (Barbosa et al. 2007). In female barn swallows (*Hirundo rustica*),

concentrations of HSP60 and HSP 70 in parasite (microfilaria, etc.) infected birds were significantly higher than parasite-free birds (Merino et al. 2002).

There were only a few studies showing the HSP responses in vertebrates during recovery period after being stressed, and the concentration responses of different HSPs were not consistent either (Inoue et al. 2009, Uma et al. 1998, Bag 1983, Yu and Bao 2008, Soleimani et al. 2011). Therefore we measured the concentration responses of HSPs during the recovery period for comparisons as well.

In this study, we assessed the changes in concentrations of the three major heat shock proteins of wild-caught house finches in response to multiple, sequential and distinct stresses. We exposed wild-caught songbirds House Finches to three different stressful environments: thermal stress, disturbance stress and pathogen stress and measured the circulating levels of HSP60, 70 and 90. We compared the magnitudes and patterns of changes in concentrations of three different HSPs in red blood cells collected after exposue to these various stressful environments.

## Materials and Methods

### *Field Sampling*

Two teams of researchers captured male house finches on 11, 12, and 13 August 2010 in Tempe and Green Valley, Arizona, approximately 135 miles apart; hereafter the samples will be referred to as "Hot-weather samples (H)". In August, the daytime high temperature on all three days of collection was 41.7$^{o}$C in Green Valley and 37.1, 39.6, 40.1$^{o}$C in Tempe (http://www.ncdc.noaa.gov/cdo-web/). Blood samples were taken immediately upon capture.

Birds were held in cages following capture and transported by van from Arizona to Auburn University, a trip that required for 29 h. The van arrived at Auburn University on 16 August 2010. Blood samples were collected immediately upon arrival and those samples were designed as "Transportation (T)". Thereafter, birds were kept in cages in an indoor animal facility at 24 $^{o}$C temperature and with ambient humidity. Two birds were put in each cage that was 1 $m^3$. Two birds were fed commercial seed and water daily. Blood samples were collected from house finches at approximately the midpoint between transport and MG infection (9 and 10 September 2010), and these samples are referred to as "Recovery period" (R). On 7 October 2010, all birds were infected by MG *(Mycoplasma Gallisepticum)* by introducing MG isolate to both eyes (Farmer 2006). Blood samples were collected after fourteen days after inoculation (Farmer 2006). Those samples were referred to as the "MG infection (MG)" group.

Blood samples were taken from each bird and stored on ice within 1 hr of capture. The whole blood samples were centrifuged at a speed 13000 rpm for 5 min at the end of each day and the packed red blood cells were stored on liquid nitrogen in the field and in a -80 $^{o}$C in the lab.

We assigned 60 house finches to the sequential stress but because of incomplete sampling, only 25 birds that had complete samples for all treatments were used in data analysis.

*Western blot*

We performed a Western Blot to quality the concentrations of blood proteins following the protocol of Tomas et al. (2004), including blood sample preparations, running gels to separate three heat shock proteins and using beta-actin protein (internal control), transferring proteins blots to membranes, incubating proteins in primary and secondary antibodies, and exposing the membranes to X-ray films in dark room. Gel analysis procedures were also the same as we described in Chapter 1 using UN-SCAN-IT version 8.1, gel version digitizing software.

Concentrations of both heat shock proteins and internal control based on the values of total pixels of blots in scanned images.

*Statistic analysis*

HSP expression was compared between stressors using a repeated measure general linear model (GLM) procedure in SAS software version 9.2. Means are reported for each HSP, controlling for within-subject effects. Stand errors are given.

**Results**

HSPs varied in responses to the four treatments (refer to Table 1). HSP90 did not show different concentrations between H, T and R groups (H-$\bar{x}$=0.97±0.17, T-$\bar{x}$=0.84±0.11, and R-$\bar{x}$=0.85±0.24). However H, T and R groups all had significant different HSP concentrations with MG group (MG-$\bar{x}$=2.52±0.27) (Figure 1, a), which also showed the highest concentration levels of HSP90. HSP70 did not show significant difference between H and T group (H-$\bar{x}$=2.80±0.25 and T-$\bar{x}$=2.70±0.29). Concentration of HSP70 was highest in MG group and lowest in R group (R-$\bar{x}$=1.63±0.31 and MG-$\bar{x}$=5.29±0.29; Figure 1, b). For HSP60, there was no significant difference in concentrations between T and R group (T-$\bar{x}$=0.76±0.07 and R-$\bar{x}$=0.66±0.09). Concentration of HSP60 was highest in MG group and lowest in H group (MG-$\bar{x}$=1.76±0.14 and H-$\bar{x}$=0.43±0.06; Figure 1, c).

**Discussion**

We observed that the different environmental stressors that we examined had different effects on concentrations of each HSP. Comparing with cool weather, as we showed in a previous study (Fu Chapter 1), concentrations of HSP70 and HSP60 but not HSP90 were

significantly induced by hot weather. We concluded that HSP70 was more sensitive and reliable to be indicator of thermal stress of wild male house finches (Fu Chapter 1).

We observed that each HSP showed a distinctive pattern of responses to the set of stressors to which birds were subjected. Moving birds a long distance in a vehicle only affected HSP90 and HSP70. Road transportation is very stressful on livestock (Adenkola and Ayo 2010), causing 40% of "dead on arrivals" of broiler chickens in UK (Mitchell and Kettlewell 1998). The time that chickens can bear the transportation stress is 2-12 h (Mitchell and Kettlewell 1998). Previous studies show that 4h transportation increased HSP70 concentrations of domestic broiler chicken (Al-Aqail and Zulkifli 2009) but total protein contents in plasma were not significantly effected by 18 vs. 1h transportation in domestic broiler chicken (Pijarska et al. 2006, Yalcin et al. 2004). The 29 h transport to which we subjected House Finches is much longer than of the transport time to which broiler chickens are subjected to. It is possible that HSPs concentrations were induced in the first couple hours in a pattern typically recorded for domestic chickens. As male house finches began to adapt to this stress, however, they shifted to other physiological mechanisms instead of producing HSPs to deal with this stressful situation. Another possible explanation is that HSP90 and HSP70 require longer time than HSP60 to produce.

During the recovery period that followed transportation, when birds were housed for weeks in indoor cages, HSP90 and HSP60 concentrations did not change significantly from what they were at the end of transportation, while HSP70 concentrations dropped. This pattern of responses is similar to what in observed in *Drosophila*. Both HSP90 and HSP70 were involved in cell-recovery mechanisms from heat stress in *Drosophila* and showed elevated concentrations during a recovery period (Duncan 2005). However HSP90 and HSP70 did not show the increased concentration over the same period. HSP90 concentration increased first and stayed unchanged

until HSP70 concentration began to rise (Duncan 2005). It seems possible that although all heat shock proteins contribute to chaperone system in supervising the protein qualities to maintain the homeostasis in cells, each specific heat shock protein family has different responsibilities under different stressful conditions, which lead to inconsistent pattern of concentration responses to various stressors. In addition, concentrations of HSP70 and HSP60 were still higher than the no-thermal stress treatments, probably due to the cage-stress that birds were experiencing when they were kept in cages in room temperature during the recovery period.

HSPs have been demonstrated to be highly sensitive to viral and bacterial infection. We observed that the highest concentrations of all three HSPs—HSP90, HSP70 and HSP60—were achieved following MG infection. MG is an endemic House Finches pathogen that has been infecting eastern House Finches since 1994 (Luttrell et al. 1998, Grodio et al. 2012). MG is stronghly pathogenic to House Finches (Roberts et al. 2001, Farmer et al. 2002), and MG infections are often fatal. Many symptoms are associated with MG infection, including severe eye lesions (Grodio et al. 2012) and high motility (Nolan et al. 2004) that can eventually lead to reduced population density of house finches. Abilities to resist MG were directly reflected by the immune system of vertebrates hosts (Jenkins et al. 2008). Both HSP90 and HSP70 were demonstrated to involve in innate and adaptive immunity of vertebrates to react with viral antigens, intracellular bacterial antigens and tumor antigens (Srivastava 2002). Our study is the first to show the MG infection effects on HSPs based on protein expression concentrations. Especially for HSP90, our result is consistent with previous studies in house finches that HSP90 can be highly induced by MG infection (Wang et al. 2006, Bonneaud et al. 2011).

Relative levels measured on low-stress, cool-weather conditions, HSP90 did not change concentrations significantly in hot weather, transportation and recovery period. Only MG

infection caused a significant change in HSP90. HSP70 kept the elevated concentrations in both hot weather and transportation treatments, and slightly declined when birds were housed in aviary at room temperature. Concentrations of HSP70 reached its relative maximum after MG infection treatment. With regard to concentration responses of HSP60, it showed increasing pattern across four stressful environments sequentially. And HSP60 had its relative maximum concentrations after MG infection treatment similar to HSP90 and HSP70.

HSP70 and HSP60 are more involved in protein folding-procedures, including rescuing denatured proteins and escorting unfolded proteins to tertiary structures (Malthews 1992, Beckman et al. 1992, Deane and Norman 2010, Wang et al. 2010) while HSP90 mainly function in signal transduction after stresses (Pałyga and Kozłowski 2008). It appears that HSP90 did not respond to environmental challenges until the birds were threatened by MG infection, which activated the signal pathway to induce concentrations of HSP90 significantly. Based on the results of our study, we conclude that HSP90 can be a potential indicator of pathogen infection stress. HSP70 increased significantly in hot weather; and then declined in the recovery period. Apparently, birds kept producing HSP70 to face challenges during hot weather and transportation, so the recovery period seems like an intermission for HSP expression. Just like HSP90, HSP70 showed its relative maximum concentrations after MG infection. Because HSP60 functions to help unfolded proteins to reach senior structures, it was not as sensitive as HSP70 to response to stresses with elevated concentrations. As birds moved from one stressful situation to another, the amount of denatured proteins in the birds grew. Denatured proteins were unfolded by HSP70, and then those unfolded proteins were assigned to chaperone HSP60 again.

In summary, our results showed that HSP90, HSP70 and HSP60 all had significant different concentrations across four treatments. For specific HSP, each had different patterns of responses

across the sequential treatments. However for all HSP MG infection induced the highest concentrations. And we believe that the among the four conditions, hot weather, transportation, recovery period and MG, HSP concentration responses of male house finches were the fiercest to MG infection.

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**Table 2,** Mean HSPs levels *(±SE)* of male House Finches in four separate conditions including H (Hot), T (Transportation), R (Recovery period) and MG (MG infection). F-value and p-value based on GLM procedure of between group effects were listed.

| | H | P | R | MG | $F_{(3,81)}$ | p-value |
|---|---|---|---|---|---|---|
| HSP90 | *0.97±0.17* | *0.84±0.11* | 0.85±*0.24* | 2.52±*0.27* | 17.01 | <0.0001 |
| HSP70 | *2.80±0.25* | *2.70±0.29* | 1.63±*0.31* | 5.29±*0.29* | 24.64 | <0.0001 |
| HSP60 | 0.43±0.06 | 0.76±0.07 | 0.66±0.09 | 1.70±0.14 | 40.02 | <0.0001 |

p-values (α=0.05): Significant differences of each specific HSP concentrations across four treatments

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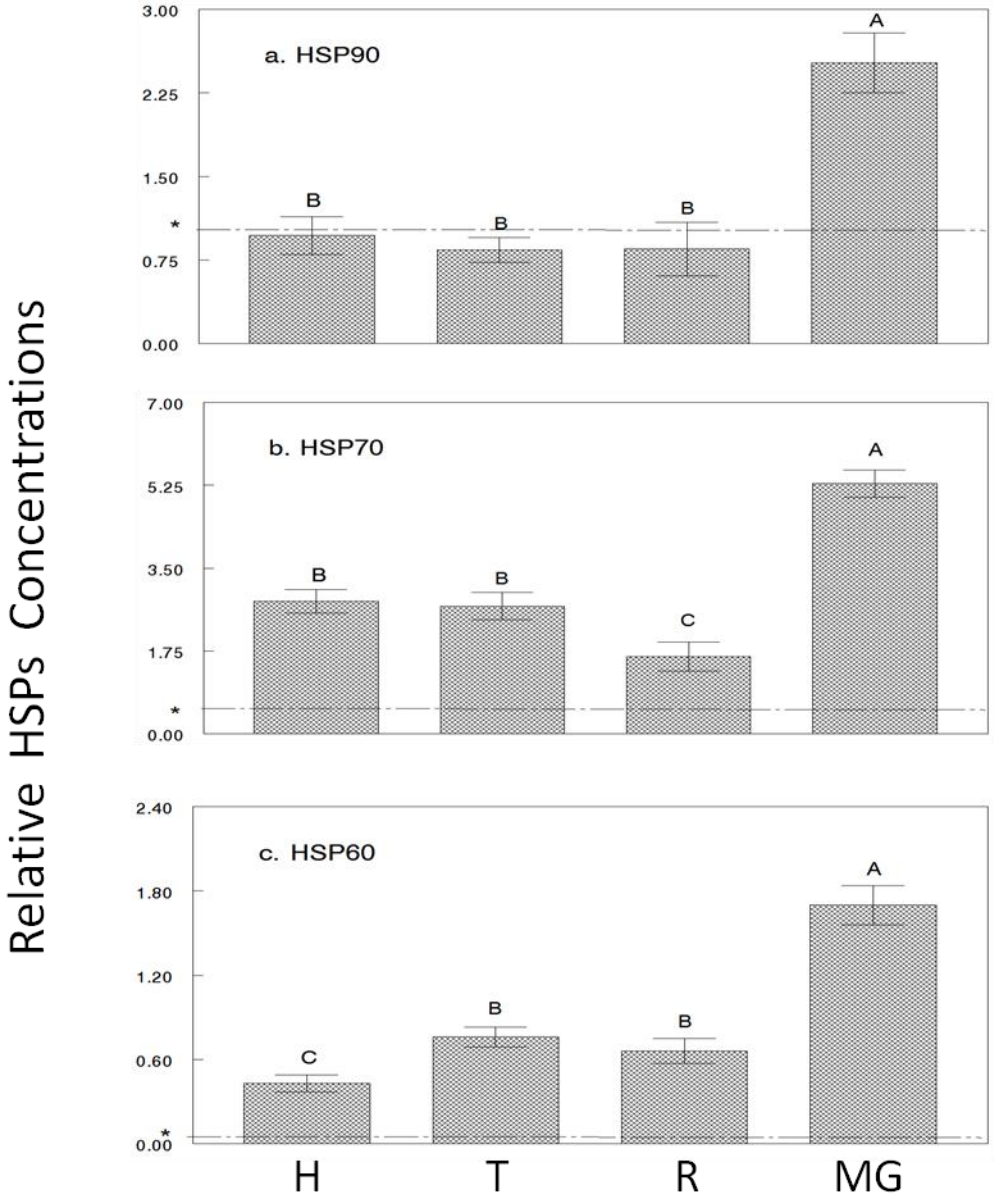
**Figure 3** Comparison of expression concentrations of HSPs in the blood of male House Finches that went through four conditions sequentially including hot weather, transportation, recovery period and MG infection (a. HSP90 *=1.04; b.HSP70: *=0.64; c.HSP60: *=0.04). Same letter from different treatments showed that there was no significant difference of specific HSP concentration between those conditions. Letter A showed the highest concentration of specific HSP across four treatments while letter C showed the lowest. The straight line with sigh "*" showed the mean concentrations of HSPs in cool weather, which were demonstrated in previous study. Relative intensities of HSPs were total pixel of blots on membranes and then were adjusted by internal control.

**Figure 3**


Relative HSPs Concentrations
a. HSP90
3.00
2.25
1.50
0.75
0.00
A
B
B
B
b. HSP70
7.00
5.25
3.50
1.75
0.00
A
B
B
C
c. HSP60
2.40
1.80
1.20
0.60
0.00
A
B
B
C
H
T
R
MG