

Changes in concentrations of circulating heat-shock proteins in House Finches in response to different environmental stressors

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ABSTRACT. Heat-shock proteins (HSP) are molecular chaperones that play key roles in the maintenance of cellular homeostasis under variable environmental conditions. Although HSP are frequently used in studies of wild vertebrates as indicators of stress, no one to date has assessed responses of HSP60, HSP70, and HSP90 in the same species to different environmental stressors. We studied changes in the circulating concentrations of HSP60, HSP70, and HSP90 in wild-caught House Finches (*Haemorrhous mexicanus*) in response to multiple and sequential stressful environments, including high temperatures, transportation, and pathogen exposure. House Finches sampled during a period of low-environmental stress with moderate ambient temperatures had low levels of HSP60 and modest levels of HSP70 and HSP90 compared to birds sampled during a presumably more stressful period with high temperatures. After exposure to high-ambient temperatures, transportation in a vehicle, and exposure to *Mycoplasma gallisepticum*, captive finches were found to have increasingly higher levels of HSP60. HSP70 tended to rise in response to each stressor, but to drop in the weeks between stress challenges. HSP90 levels increased significantly only in response to pathogen challenge. Our observations suggest that HSP60 and HSP70 are indices of a range of stressors in House Finches, whereas HSP90 primarily reflects health state.

RESUMEN. Cambios en la concentración de proteínas producidas por estrés, causado por el calor (HSP), en *Haemorrhous mexicanus* como respuesta a diferentes estresantes ambientales

Proteínas que se producen por el estrés causado por el calor (HSP) son chaperones moleculares que juegan un papel clave en el mantenimiento de la homeostasis celular bajo diferentes condiciones ambientales. Aunque las HSP son utilizadas con frecuencia en estudios de vertebrados silvestres como indicadores de estrés, hasta el momento nadie ha tratado de determinar la respuesta de HSP60, HSP70 y HSP90, en la misma especie, bajo diferentes condiciones ambientales estresantes. Estudiamos los cambios en las concentraciones de HSP60, HSP70 y HSP90, en el sistema circulatorio de individuos de *Haemorrhous mexicanus* silvestres capturados, como respuesta a múltiples estresantes ambientales, incluyendo altas temperaturas, transportación y exposición a patógenos. Los pinzones muestreados durante un período de poco estrés ambiental, con temperaturas ambientales moderadas, mostraron bajos niveles de HSP60, y niveles modestos de HSP70 y HSP90, al ser comparados con aves muestreadas, presumiblemente, durante periodos más estresantes (altas temperaturas). Luego de que las aves cautivas fueran expuestas a altas temperaturas, a transporte en vehículos de motor y a *Mycoplasma gallisepticum*, se encontraron en estas altos niveles de HSP60. El HSP70 tendió a incrementar en respuesta a cada estresante, pero se redujo en las semanas entre la aplicaciones del estresor. Por su parte el HSP90 solo aumentó, significativamente, en respuesta a los patógenos. Nuestras observaciones sugieren que el HSP60 y HSP70 son índices de una gama de estresantes en individuos del pinzón estudiado, mientras que HSP90 refleja el estado de salud del individuo.

Key words: *Haemorrhous mexicanus*, heat stress, *Mycoplasma gallisepticum*, pathogen stress

A variety of environmental factors, including extreme temperatures, toxins, oxidative stress, pH imbalance, and disease, can disrupt core cellular processes. Animals respond to such challenges with a number of homeostatic mechanisms, and the so-called heat-shock response

is among the core stress-response mechanisms (Craig 1985, Feder and Hofmann 1999). The heat-shock response involves heat-shock proteins (HSPs), a functional grouping of several families of chaperone molecules (Lindquist 1986, Sørensen et al. 2003). These molecules are referred to as chaperones because their primary role is to accompany proteins and ensure their proper function. They are called HSPs because

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thermal extremes challenge protein integrity and trigger a strong HSP response (Sørensen et al. 2003). Many different environmental stressors, however, can activate HSPs (Lindquist 1986, Sørensen et al. 2003). Even under optimal conditions, some HSPs are present in cells, assisting in the folding of newly created proteins, correcting folding alignments of active proteins, and targeting destruction of proteins that cannot be repaired (Kregel 2002). Because of their responsiveness to stressful environments, HSP levels can potentially serve as indices of stress in vertebrates (Lewis et al. 1999, Iwama et al. 2004, Herring and Gawlik 2007).

When animals are subjected to stressful situations, the concentrations of HSPs in tissue can increase to 20% of total cellular proteins (Bhardwaj et al. 2012), but not all HSPs necessarily respond in the same manner to all stressors. To properly employ HSPs as indices of stress, understanding how different families of HSPs respond to different types of stressors is critical (Fader et al. 1994). Three major families of HSPs have been identified in vertebrates—HSP60, HSP70, and HSP90—and each of these families of HSP has been used as a measure of stress in various studies of vertebrates (Sørensen et al. 2003, Sørensen 2010, Salway et al. 2011). HSP70, the most structurally conserved and ubiquitous HSP, regulates folding and structural maintenance of proteins (Kriegenburg et al. 2012). In some fish and reptiles, HSP70 is more sensitive to environmental perturbations than HSP60 or HSP90 (e.g., Ulmasov et al. 1992, Fader et al. 1994, McMillan et al. 2011) and, consequently, HSP70 has been proposed to be a particularly sensitive biomarker of stress (Lewis et al. 1999). HSP60 assists primarily in protein folding and is highly responsive to hypothermia (Herring and Gawlik 2007), but HSP60 also plays a key role in the assembly of proteins within cells and in the immune system of vertebrates (Young 1990). HSP60 is the HSP most widely assayed in studies of stress in birds (e.g., Merino et al. 1998, 2002). HSP90 functions somewhat differently than HSP60 or HSP70. Rather than maintaining the integrity of single proteins, HSP90 appears to facilitate the assembly and maintenance of protein complexes (Makhnevych and Houry 2012). In studies of lab animals, increased levels of HSP90 are associated with hypothermia tolerance, cell proliferation, cell cycle control, and immune responsiveness (Csermely et al. 1998, Young

et al. 2001). HSP90 is infrequently assayed in ecological studies.

Given that HSP60, HSP70, and HSP90 are known to facilitate different aspects of homeostatic control, we predicted that these three families of HSP would show distinctive patterns of response to different environmental challenges. We refer to such environmental challenges as stressors that inflict stress on birds (Sohn et al. 2012). Biologists commonly use the term stress to describe both the physiological response to a stressor as well as the stressor itself (Romero et al. 2009). We use the term stress to mean an environmental condition that disrupts the functionality of the organism (*sensu* Badyaev 2005, Hill 2011).

To test the hypothesis that different HSPs respond in different ways to different types of stress, we assessed changes in concentrations of HSP60, HSP70, and HSP90 in House Finches (*Haemorrhous mexicanus*) in response to a series of stressful environments. We first compared levels of circulating HSPs in the same population of wild House Finches sampled during a period of high stress (feather molt and high temperatures) in late summer and in a period of comparatively low stress (no molt and moderate temperatures) in the fall. Second, following initial sampling, we transported summer-caught House Finches to an aviary for an experiment involving pathogen infection. Thus, over a 3-mo period, these finches were exposed to two additional stressors: prolonged transportation in a vehicle while confined to small cages and infection by a pathogenic bacterium (*Mycoplasma gallisepticum*; hereafter MG). To gain a better understanding of how HSPs respond to different stressors in songbirds, we took blood samples from finches following each of these potentially stressful events and compared concentrations of circulating HSP60, HSP70, and HSP90 across sampling points.

METHODS

Sampling free-living House Finches.

We captured wild House Finches at backyard feeding stations in southeastern Arizona (AZ). All birds collected for our study were hatching-year males. We determined sex by the presence of carotenoid-colored feathers on the breast and throat, and determined that birds were hatching year by the presence of streaky juvenile feathers on the throat and breast (Hill 2002). First,

we captured finches in Green Valley, AZ, and Tempe, AZ, from 11–13 August 2010. We then caught finches in Tempe, AZ, on 10, 17, 18, and 29 November 2011. In both August and November, captured birds were resident birds hatched in the region as indicated by banding studies in AZ (Oh and Badyaev 2010). Green Valley and Tempe are 216-km apart, but have similar habitats and weather patterns. Moreover, House Finches at the two sites belong to one subspecies (Moore 1939). Thus, we anticipated no differences in physiological responses of finches from the two populations, and we considered parallel work at the two locations to represent a single collection effort. Nevertheless, we tested for statistical differences between populations before pooling individuals between sites (see Results).

On the 3 d of collection in August, daytime high temperatures were 37.1, 39.6, and 40.1°C in Tempe and 41.7°C each day in Green Valley (<http://www.ncdc.noaa.gov/cdo-web/>), with low temperatures between 26.2°C and 27.1°C. These temperatures exceed the highest temperatures experienced by House Finches throughout most of the eastern and western range of the species (<http://www.ncdc.noaa.gov/cdo-web/>). We refer to samples taken under these conditions ($N = 82$) as summer samples, and we focus on high-ambient temperature as a driver in patterns of HSPs. However, birds sampled in August were undoubtedly subjected to other stressors. Importantly, birds sampled in August were undergoing prebasic molt whereas birds sampled in November were not.

To establish a baseline of HSP levels under conditions of low stress, we then sampled hatching-year male House Finches in November 2011 during periods of cooler weather, with daytime high temperatures of 21.6°C, 26.1°C, 25.0°C, and 23.2°C, respectively. Low temperatures on collecting dates were 13.2°C, 14.1°C, 13.9°C, and 13.7°C, respectively. Temperatures on these days should be near the thermoneutral temperature for a temperate granivorous songbird (Rezende et al. 2001). We refer to samples taken under these conditions ($N = 25$) as fall samples. In both sampling periods, all birds were non-breeding with minimal gonadal development and, because all were hatch-year birds, no birds in our study had bred previously.

In both August and November, traps were checked hourly, and birds in traps remained calm

until a trap was approached by a human. There were typically 10–20 individuals in the wire-basket trap (Hill 2002) when checked. Birds in the trap were quickly grabbed by hand, and each was placed in a brown paper bag, which shielded birds from visual stimulation and allowed them to stand in a comfortable posture. Within 1 h of removal from the trap, a blood sample was taken from each bird by puncturing a brachial vein with a 27-gauge needle. Blood was drawn into a heparinized microhematocrit tube and transferred to an Eppendorf tube (see below for final blood processing). Here and during all subsequent sampling, blood samples were chilled on ice immediately upon collection. Within 6 h of collection, blood was spun at $13,000 \times g$ for 5 min and the serum separated from packed red blood cells. Packed red blood cells were stored on liquid nitrogen in the field and at -80°C in the lab.

Approximately 200 μl of blood was collected from each bird and they were then placed back in the paper bag. Birds were transported by car to hotel rooms in Tucson or Tempe, AZ, where they were held in groups of 10 in small cages (0.75 m \times 0.5 m \times 0.5 m) with ad libitum access to food (sunflower seeds) and water and with a natural photoperiod. Cages were covered with translucent white cloths so birds did not perceive human activity. On the afternoon of 13 August 2010, all birds were consolidated in a room in Tucson.

Transportation stress. One hour before sunrise on 14 August 2010, cages containing House Finches ($N = 101$) were carried to a van and the birds were driven to Auburn, Alabama. This entailed 29 h of driving. From midnight until 05:00, researchers stopped to sleep, during which time the birds were left in the van with partially open windows. The van was air-conditioned when running during daylight hours, and ambient temperature was $\sim 28^\circ\text{C}$. Researchers took brief breaks for meals totaling 4 h, so total time in the van was 38 h. Immediately upon arrival in Auburn on 15 August, a blood sample was taken from each bird following protocols described above (hereafter, transport-stress sample). The birds were then held in pairs in small cages (0.5 m³) in indoor rooms for 8 weeks, until infection experiments began (see below). Rooms were maintained at $\sim 24^\circ\text{C}$ and with humidity at $\sim 60\%$. At approximately the halfway point between the end of transport and

pathogen infection (9 and 10 September 2010), we drew another blood sample from each bird (hereafter, cage sample).

Pathogen infection. On 7 October 2010, birds ($N = 56$) were inoculated with MG by dropping 10 μ l of MG-infected media into each eye following the protocols in Farmer et al. (2002). This media was from a stock culture containing $\sim 10^3$ – 10^6 color-changing units/ml of an MG field isolate collected in Auburn, Alabama, in January 2007. MG infects the respiratory system and conjunctiva of the eyes of House Finches. After 14 d, all birds showed symptoms of mycoplasmosis, as indicated by swelling of eye conjunctiva, and a blood sample was taken from each infected bird (hereafter, MG sample).

HSP measurement. We followed methods described in Merino et al. (1998) and Tomás et al. (2004) for blood cell processing and western blotting except that we included an internal control for each gel. We used an antibody to a well-characterized housekeeping gene, β -actin, to normalize the relative expression of HSPs between gels. Briefly, pelleted red blood cells were resuspended in 100–200 ml of nanopure water and briefly sonicated to disrupt cells and release their contents. Samples were centrifuged (13,000g) for 20 min, and the supernatant was transferred to a new tube.

Protein concentration of the supernatant was determined with the Quick Start™ Bradford Assay (BioRad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE in 10% gels at 200 V for 1 h and transferred to PVDF (polyvinylidene fluoride) membranes via a semidry electrophoretic transfer cell at 20 V for 30 min. Membranes were washed three times for 5 min each with PBS (phosphate buffered saline) containing 0.05% Tween-20 (wash buffer), followed by blocking with 5% nonfat powdered milk in wash buffer for 90 min. Primary monoclonal antibodies for HSP60 (clone LK-1, Abcam, Cambridge, MA), HSP70 (clone N27F3-4, Abcam, Cambridge, MA), and HSP90 (clone AC-16, Sigma-Aldrich, St. Louis, MO) were diluted to 1/1333, 1/4000, and 1/1333 in wash buffer, respectively. Monoclonal anti- β -actin (clone AC-15) was diluted to 1/4000 in wash buffer to be used as internal control for each of the HSP. Membranes were incubated with primary antibodies for 3 h, followed by three 5-min washes. The secondary antibody (HRP-

conjugated, antimouse) was diluted to 1/5000 in wash buffer before being added to membranes for 30-min incubation. Membranes were then washed three times before visualization onto photographic x-ray film with Amersham ECL select (GE Healthcare, Pittsburgh, PA) according to manufacturers protocols. X-ray films were scanned using a Canon PIXMA, MP560 and bands were quantified with UN-SCAN-IT software (v. 8.1). Immunoreactivity was calculated as the area of a band multiplied by intensity after normalizing for intensity of within-gel standards. We used immunoreactivity (arbitrary units) as our estimate of the concentration of each HSP (following Tomás et al. 2004).

Statistics. All analyses were completed using SAS 9.2 (SAS Institute 2002–2008). Circulating HSP concentrations for birds collected during summer in Tempe and Green Valley were compared using a *t*-test. Satterthwaite's method was applied when the variance was unequal between groups (Cody and Smith 1991). We found no difference between Tempe and Green Valley birds in concentrations of any HSP (Table 1) and thus data were combined for additional comparisons of HSP reactivity during a time of high-environmental stress. A *t*-test and Satterthwaite's method (where applicable) were also used to compare HSP concentrations for birds collected during summer and late fall (i.e., times of high- and low-environmental stress). Finally, a generalized linear mixed model (Littell et al. 2006) was used to examine the effect of transport, cage, and disease stressors on HSP concentrations across the same individuals. Individual identification number was included as a random effect to control for repeated measures. Cage and number of cage-mates were also included as covariates in the initial models, but were later removed because neither had a significant impact on HSP levels ($P > 0.13$). Comparisons of the least-squares means were obtained with the DIFF option in SAS.

RESULTS

Levels of HSP60 and HSP70 in response to all stressors were greater than the baseline levels established in fall sampling (Fig. 1). Circulating levels of HSP60 in birds following transport stress and when maintained in cages were significantly higher than levels measured in birds in the field in the summer and significantly

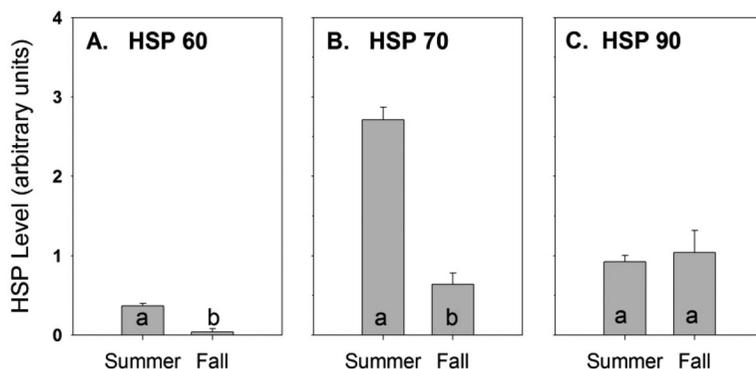


Fig. 1. Mean levels (\pm SE) of circulating HSP60, HSP70, and HSP90 as estimated by relative immunoreactivity (arbitrary units) for male House Finches sampled in Arizona in hot weather in the summer and cooler weather in the fall. Letters within each bar indicate results of pairwise comparisons, and means for groups with different letters are statistically different.

higher still when birds were infected with MG (Table 1, Fig. 2A). HSP70 levels dropped significantly, but remained above baseline, as birds were held in cages between transport and pathogen challenges and then rose to the highest concentrations in response to MG infection (Fig. 2B). HSP90 levels among birds sampled in summer heat, following transportation, or after an extended period in a cage did not differ (Fig. 2C), but HSP90 levels were significantly higher in birds infected with MG (Fig. 2C).

DISCUSSION

An important caveat to our study is that we were unable to include controls for our treatments. We attempted to establish a baseline for all treatments (fall samples), but baseline observations were made at a different season than all other values and the birds were one to several months older than birds in other treatments. Thus, although we provide a reasonable interpretation for the HSP responses, factors other than the stressors on which we focused might have contributed to the observed patterns.

As predicted, we documented distinctive patterns of responsiveness by different HSPs. We took the circulating levels of HSP in wild House Finches captured during the fall in cool weather and when they were neither breeding nor molting as a baseline to which stress-induced changes could be compared. None of the birds in the fall sample had detectable parasites or signs of illness, but we were blind to endoparasitic

infections that did not induce conspicuous symptoms. HSP60 levels in the fall were at levels so low they could barely be detected and levels of both HSP70 and HSP90 increased from this fall sampling level when birds were later subjected to stressors, supporting the contention that the cool-weather sampling in the fall represented a reasonable baseline for comparison with unambiguously stressful conditions. Such baselines are rarely established in ecological studies, but provide an important reference when invoking a stress response by HSPs (Fader et al. 1994).

The three HSPs showed distinctive responses to the high temperatures experienced by House Finches in our study. In cool fall weather, levels of HSP60 were low and nearly undetectable, but, with higher summer temperatures, HSP60 levels were higher and easily detectable. HSP70 circulated at relatively high levels during both summer and fall periods, but levels were significantly higher during the summer. Levels of HSP90, in contrast, did not differ significantly in the summer and fall. Many animals show physiological adaptation to repeated or long-term exposure to high-ambient temperatures (Eichna et al. 1950), leading, for example, to less extreme increases in HSP70 levels in response to high temperatures in domestic chickens (*Gallus gallus domesticus*; Liew et al. 2003). We might have expected such physiological adaptation in House Finches in our study given that they had been exposed to high temperatures throughout the summer leading up to August sampling. At high temperatures, however, such physiological

Table 1. Relative levels (mean ± SE) of HSP60, HSP70, and HSP90 under different environmental conditions, including different locations (location), different weather conditions (temperature), and different stressors. Birds were collected simultaneously in Tempe and Green Valley, AZ. Because means from these locations were not statistically different, data were collapsed into the summer group. Results of statistical comparisons are given, including *t*-tests for location and temperature comparisons and mixed-models for stressor comparisons.

	Location			Temperature		Stressor			
	Tempe, AZ	Green Valley, AZ	Summer	Summer	Fall	Summer	Transport stress	Cage stress	Pathogen infection
HSP60	0.44 ± 0.05 <i>t</i> ₇₀ = 1.6, <i>P</i> = 0.12	0.33 ± 0.05	0.38 ± 0.29 ^a <i>t</i> _{69.7} = 6.9, <i>P</i> < 0.0001	0.04 ± 0.17	0.38 ± 0.04	0.80 ± 0.05 <i>F</i> _{3,61} = 97.1, <i>P</i> < 0.0001	0.68 ± 0.05	1.72 ± 0.09	
HSP70	2.95 ± 0.22 <i>t</i> ₇₂ = 1.7, <i>P</i> = 0.10	2.42 ± 0.22	2.74 ± 0.16 ^a <i>t</i> _{79.3} = 9.7, <i>P</i> < 0.0001	0.64 ± 0.14	2.71 ± 0.16	2.40 ± 0.17 <i>F</i> _{3,68} = 46.1, <i>P</i> < 0.0001	1.56 ± 0.16	4.68 ± 0.22	
HSP90	1.03 ± 0.12 <i>t</i> ₈₁ = 1.8, <i>P</i> = 0.08	0.74 ± 0.11	0.90 ± 0.08 ^a <i>t</i> _{22.5} = 0.5, <i>P</i> = 0.64	1.04 ± 0.28	0.92 ± 0.09	1.04 ± 0.10 <i>F</i> _{3,73} = 10.5, <i>P</i> < 0.0001	1.20 ± 0.17	1.90 ± 0.18	

^aApplied Satterthwaite's method for unequal variance.

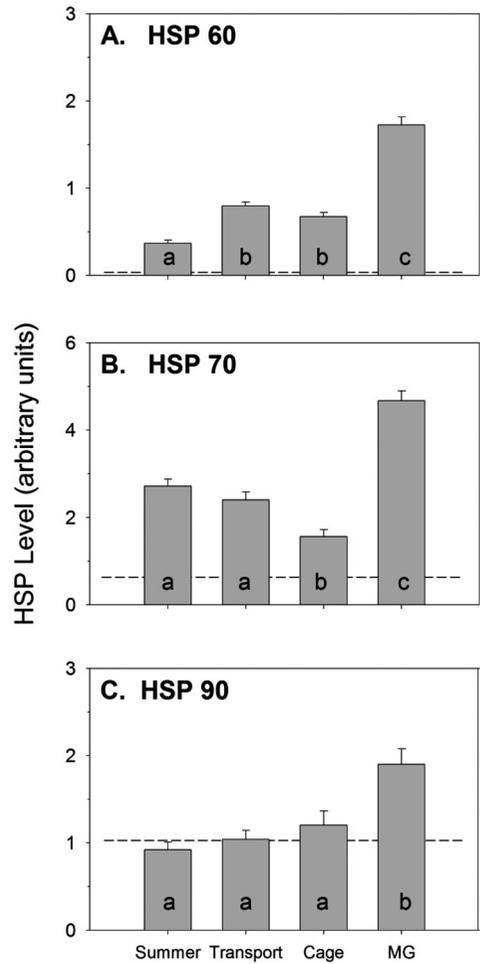


Fig. 2. Mean levels (±SE) of circulating HSP60, HSP70, and HSP90 in wild-caught male House Finches as estimated by relative immunoreactivity (arbitrary units) in response to different stressors, including hot summer weather, transportation stress, and MG (*Mycoplasma gallisepticum*) infection. Cage treatment was a between-stress period where birds were held in indoor cages and subject to no specific stressors. Concentrations of HSPs under cooler-weather conditions (dashed lines) are provided for qualitative comparison. Letters within each bar indicate results of pairwise comparisons, and means for groups with different letters are statistically different.

acclimatization may not be possible because protein integrity simply cannot be maintained without increased levels of HSP (Givisiez et al. 1999). Our observations suggest that the high temperatures experienced by House Finches

during the summer required elevated levels of HSP60 and HSP70 to maintain system functionality.

We cannot with certainty attribute differences in HSPs between summer and fall sampling periods exclusively to the effects of high-ambient temperatures, although we consider temperature to be the likely driver of changes in HSPs. All birds included in our study in both summer and fall sampling periods were hatched in the year we sampled them, so they had never developed functional gonads. Reproductive status or experience was not a confounding factor in this study. There were, however, other differences between the summer and fall sampling periods besides temperature. Birds sampled in the fall were about 4 mo older than birds sampled in August and developmental changes could have occurred in these young birds over that period. Moreover, in August, all male House Finches sampled were molting, whereas none of the birds was molting in November. Molt requires rapid production of proteins for feather construction (Cornelius et al. 2011) and would be expected to induce higher levels of HSPs. Thus, stress from protein production during molt likely added to stress from high-ambient temperatures, exacerbating the contrast of low-stress conditions in fall versus high-stress conditions in summer. However, in a study of molt/breeding overlap in Pied Flycatchers (*Ficedula hypoleuca*), Morales et al. (2007) found significantly lower HSP60 among birds with molt/breeding overlap compared to individuals without such an overlap. These observations suggest that molt may not have been a serious confounding effect in our study. We can identify no stress factor that was likely higher for House Finches in Arizona in fall than summer.

The three HSPs showed distinct patterns of response across the sequential stress challenges. HSP60 rose significantly with transportation stress, did not drop significantly during the 2-week recovery period, and then more than doubled following MG infection. These results suggest that levels of HSP60 do not recover quickly after stressful events in House Finches. Concentrations of HSP60 were almost too low to measure during cool weather, but then rose in steps, increasing significantly through hot weather, transportation, and pathogen challenges. This pattern suggests that the sequential stressors had a cumulative effect on levels of

HSP60. HSP60 is the most commonly measured HSP in studies of wild birds and a range of stressors, including nestling crowding, parasites, and nutritional stress (Merino et al. 1998, 2002, del Cerro et al. 2010, Martínez-de la Puente et al. 2011), have been shown to cause or be associated with an increase in the levels of HSP60. Our observations support these previous studies that show that HSP60 is responsive to a wide range of stressors in songbirds.

In contrast to HSP60, levels of HSP70 in our study showed no change from the already-elevated levels measured in hot weather following transport stress, but then dropped significantly during the recovery period before they tripled in concentration following MG infection. These results suggest that HSP70 may have greater capacity for short-term recovery in songbirds than HSP60. In a study of wild Common Eiders (*Somateria mollissima*; Bourgeon et al. 2006), levels of both HSP70 and HSP60 rose through the incubation period, presumably in response to stress induced by loss of body heat and lack of food. However, in a study of Blue Tits (*Parus caeruleus*), levels of HSP60 increased with increasing levels of blood parasite infection, whereas HSP70 showed no significant change (Tomás et al. 2005). Conversely, in a study where Pied Flycatchers were exposed to predators, levels of HSP70 increased whereas levels of HSP60 showed no significant response (Thomson et al. 2010). The differences we observed in the responses of HSP70 and HSP60 corroborate the results of previous studies that indicate that the responses of these two HSPs are distinct.

Finally, HSP90 showed no significant response to any stressors until pathogen exposure. In response to MG infection, HSP90 more than doubled in concentration. Although rarely quantified in ecological studies, our results suggest that HSP90 may be a particularly valuable indicator of pathogen infection because it responded specifically to this form of stress. Interestingly, in a study of gene expression in the spleens of House Finches infected with MG, HSP90 was the only HSP that was expressed significantly differently in the spleens of infected versus control finches (Wang et al. 2006, Bonneaud et al. 2011). Because it functions in the assembly and maintenance of protein complexes rather than individual proteins (Makhnevych and

Hourly 2012), HSP90 may play a particularly important role in the assembly of protein complexes such as gamma globulins that are upregulated and mobilized in response to invasion by pathogens.

In many studies of confined birds and other animals, individuals may be subjected to the same sort of sequential stressors as the birds in our study, for example, being captured, handled for measuring and, perhaps, collecting blood samples, transported in some sort of container, and kept in cages with a novel diet. Many studies begin physiological monitoring with the onset of captivity, ignoring previous treatments. Our data suggest that levels of HSP70 and HSP90 can recover, at least to a degree, within a few weeks following a stressful event, but levels of HSP60 may take longer to recover. Such differences in patterns of recovery should be taken into consideration when assessing levels of HSP in studies of wild birds.

In sum, our results suggest that changes in levels of HSP60 and HSP70 in the blood appear to be good indicators of heat stress and situational stress (transportation). However, HSP90 responded only to pathogen exposure, suggesting that this HSP would appear to be a particularly appropriate indicator of the health state of wild birds. In addition, the differences in baseline levels of the three HSP underscore the need to establish a baseline to which proposed stress responses can be compared.

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LITERATURE CITED

- BADYAEV, A. V. 2005. Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. *Proceedings of the Royal Society B* 272: 877–886.
- BHARDWAJ, N., J. DORMER, F. AHMAD, A. D. STRICKLAND, G. GRAVANTE, I. BECKINGHAM, K. WEST, A. R. DENNISON, AND D. M. LLOYD. 2012. Heat shock protein 70 expression following hepatic radio frequency ablation is affected by adjacent vasculature. *Journal of Surgical Research* 173: 249–257.
- BONNEAUD, C., S. L. BALENGER, A. F. RUSSELL, J. W. ZHANG, G. E. HILL, AND S. V. EDWARDS. 2011. Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proceedings of the National Academy of Sciences USA* 108: 7866–7871.
- BOURGEON, S., J. MARTÍNEZ, F. CRISCUOLO, Y. LE MAHO, AND T. RACLOT. 2006. Fasting-induced changes of immunological and stress indicators in breeding female eiders. *General and Comparative Endocrinology* 147: 336–342.
- CODY, R. P., AND J. K. SMITH. 1991. *Applied statistics and the SAS programming language*. Prentice Hall, Englewood Cliffs, NJ.
- CORNELIUS, J. M., N. PERFITO, R. ZANN, C. W. BREUNER, AND T. P. HAHN. 2011. Physiological trade-offs in self-maintenance: plumage molt and stress physiology in birds. *Journal of Experimental Biology* 214: 2768–2777.
- CRAIG, E. A. 1985. The heat-shock response. *CRC Critical Reviews in Biochemistry* 18: 239–280.
- CSERMELY, P., T. SCHNAIDER, C. SOTI, Z. PROHASZKA, AND G. NARDAL. 1998. The 90-kDa molecular chaperone family: structure, function, and clinical applications: a comprehensive review. *Pharmacology & Therapeutics* 79: 129–168.
- DEL CERRO, S., S. MERINO, J. MARTÍNEZ-DE LAUENTE, E. LOBATO, R. RUIZ-DE-CASTAÑEDA, J. RIVERO-DE AGUILAR, J. MARTÍNEZ, J. MORALES, G. TOMÁS, AND J. MORENO. 2010. Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in Blue Tits (*Cyanistes caeruleus*). *Oecologia* 162: 825–835.
- EICHNA, L. W., C. R. PARK, N. NELSON, S. M. HORVATH, AND E. D. PALMES. 1950. Thermal regulation during acclimatization in a hot, dry (desert type) environment. *American Journal of Physiology* 163: 585–597.
- FADER, S. C., Z. M. YU, AND J. R. SPOTILA. 1994. Seasonal-variation in heat-shock proteins (HSP70) in stream fish under natural conditions. *Journal of Thermal Biology* 19: 335–341.
- FARMER, K. L., G. E. HILL, AND S. R. ROBERTS. 2002. Susceptibility of a naive population of House Finches to *Mycoplasma gallisepticum*. *Journal of Wildlife Diseases* 38: 282–286.
- FEDER, M. E., AND G. E. HOFMANN. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61: 243–282.
- GIVISIEZ, P. E. N., J. A. FERRO, M. I. T. FERRO, S. N. KRONKA, E. DECUYPERE, AND M. MACARI. 1999. Hepatic concentration of heat shock protein 70 kD (HSP70) in broilers subjected to different thermal treatments. *British Poultry Science* 40: 292–296.
- HERRING, G., AND D. E. GAWLIK. 2007. The role of stress proteins in the study of allostatic overload in birds: use and applicability to current studies in avian ecology. *The Scientific World Journal* 7: 1596–1602.
- HILL, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in

- the House Finch. Oxford University Press, Oxford, UK.
- . 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecology Letters* 14: 625–634.
- IWAMA, G. K., L. O. B. AFONSO, A. TODGHAM, P. ACKERMAN, AND K. NAKANO. 2004. Are hsp90 suitable for indicating stressed states in fish? *Journal of Experimental Biology* 207: 15–19.
- KREGEL, K. C. 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology* 92: 2177–2186.
- KRIEGBURG, F., L. ELLGAARD, AND R. HARTMANN-PETERSEN. 2012. Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation. *FEBS Journal* 279: 532–542.
- LEWIS, S., R. D. HANDY, B. CORDI, Z. BILLINGHURST, AND M. H. DEPLEDGE. 1999. Stress proteins (HSP's): methods of detection and their use as an environmental biomarker. *Ecotoxicology* 8: 351–368.
- LIEW, P. K., I. ZULKIFLI, M. HAIR-BEJO, A. R. OMAR, AND D. A. ISRAF. 2003. Effects of early age feed restriction and heat conditioning on heat shock protein 70 expression, resistance to infectious bursal disease, and growth in male broiler chickens subjected to heat stress. *Poultry Science* 82: 1879–1885.
- LINDQUIST, S. 1986. The heat-shock response. *Annual Review of Biochemistry* 55: 1151–1191.
- LITTELL, R. C., G. A. MILLIKEN, W. W. STROUP, R. D. WOLFFINGER, AND O. SCHABENBERGER. 2006. SAS for Mixed Models SAS Institute, Inc., Cary, NC.
- MAKHNEVYCH, T., AND W. A. HOURLY. 2012. The role of Hsp90 in protein complex assembly. *Biochimica Et Biophysica Acta-Molecular Cell Research* 1823: 674–682.
- MARTÍNEZ-DE LA PUENTE, J., S. MERINO, G. TOMÁS, J. MORENO, J. MORALES, E. LOBATO, AND J. MARTÍNEZ. 2011. Nest ectoparasites increase physiological stress in breeding birds: an experiment. *Naturwissenschaften* 98: 99–106.
- MCMILLAN, D. M., D. J. IRSCHICK, AND B. B. REES. 2011. Geographic variation in the effects of heat exposure on maximum sprint speed and Hsp70 expression in the western fence lizard *Sceloporus occidentalis*. *Physiological and Biochemical Zoology* 84: 573–582.
- MERINO, S., J. MARTÍNEZ, A. BARBOSA, A. P. MØLLER, F. DE LOPE, J. PEREZ, AND F. RODRIGUEZ-CAABEIRO. 1998. Increase in a heat-shock protein from blood cells in response of nestling House Martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia* 116: 343–347.
- , ———, A. P. MØLLER, A. BARBOSA, F. DE LOPE, AND F. RODRIGUEZ-CAABEIRO. 2002. Blood stress protein levels in relation to sex and parasitism of Barn Swallows (*Hirundo rustica*). *Ecoscience* 9: 300–305.
- MOORE, R. T. 1939. A review of the House Finches of the subgenus *Burrica*. *Condor* 41: 177–205.
- MORALES, J., J. MORENO, S. MERINO, J. J. SANZ, G. TOMÁS, E. ARRIERO, E. LOBATO, AND J. MARTÍNEZ-DE LA PUENTE. 2007. Early moult improves local survival and reduces reproductive output in female Pied Flycatchers. *Ecoscience* 14: 31–39.
- OH, K. P., AND A. V. BADIYAEV. 2010. Structure of social networks in a passerine bird: consequences for sexual selection and the evolution of mating strategies. *American Naturalist* 176: E80–E89.
- REZENDE, E. L., M. V. LÓPEZ-CALLEJA, AND F. BOZINOVIC. 2001. Standard and comparative energetics of a small avian herbivore (*Phytotoma rara*). *Auk* 118: 781–785.
- ROMERO, L. M., M. J. DICKENS, AND N. E. CYR. 2009. The reactive scope model—a new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior* 55: 375–389.
- SALWAY, K. D., E. J. GALLAGHER, M. M. PAGE, AND J. A. STUART. 2011. Higher levels of heat shock proteins in longer-lived mammals and birds. *Mechanisms of Ageing and Development* 132: 287–297.
- SAS INSTITUTE. (2002–2008). SAS 9.2. SAS Institute, Inc., Cary, NC.
- SOHN, S. H., V. K. SUBRAMANI, Y. S. MOON, AND I. S. JANG. 2012. Telomeric DNA quantity, DNA damage, and heat shock protein gene expression as physiological stress markers in chickens. *Poultry Science* 91: 829–836.
- SØRENSEN, J. G. 2010. Application of heat shock protein expression for detecting natural adaptation and exposure to stress in natural populations. *Current Zoology* 56: 703–713.
- , T. N. KRISTENSEN, AND V. LOESCHCKE. 2003. The evolutionary and ecological role of heat shock proteins. *Ecology Letters* 6: 1025–1037.
- THOMSON, R. L., G. TOMÁS, J. T. FORSMAN, J. BROGGI, AND M. MONKKONEN. 2010. Predator proximity as a stressor in breeding flycatchers: mass loss, stress protein induction, and elevated provisioning. *Ecology* 91: 1832–1840.
- TOMÁS, G., J. MARTÍNEZ, AND S. MERINO. 2004. Collection and analysis of blood samples to detect stress proteins in wild birds. *Journal of Field Ornithology* 75: 281–287.
- TOMÁS, G., S. MERINO, J. MARTÍNEZ, J. MORENO, AND J. J. SANZ. 2005. Stress protein levels and blood parasite infection in blue tits (*Parus caeruleus*): a medication field experiment. *Annales Zoologici Fennici* 42: 45–56.
- ULMASOV, K. A., S. SHAMMAKOV, K. KARAEV, AND M. B. EVGENEV. 1992. Heat-shock proteins and thermoresistance in lizards. *Proceedings of the National Academy of Sciences USA* 89: 1666–1670.
- WANG, Z., K. FARMER, G. E. HILL, AND S. V. EDWARDS. 2006. A cDNA macroarray approach to parasite-induced gene expression changes in a songbird host: genetic response of House Finches to experimental infection by *Mycoplasma gallisepticum*. *Molecular Ecology* 15: 1263–1273.
- YOUNG, J. C., I. MOAREFI, AND F. U. HARTL. 2001. HSP90: a specialized but essential protein-folding tool. *Journal of Cell Biology* 154: 267–273.
- YOUNG, R. A. 1990. Stress proteins and immunology. *Annual Review of Immunology* 8: 401–420.