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Ectothermy and endothermy: evolutionary perspectives of thermoprotection by HSPs

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Summary

Living organisms respond to heat exposure by selectively expressing heat shock proteins (HSPs). Accumulation of HSPs confers thermotolerance in cell cultures and in ectotherms and is an important component of the heat shock response. This response, however, has not been directly examined in relation to different 'thermal states', namely ectothermy *vs* endothermy. By using avian development as a model system for transition from ectothermy to endothermy, we show that, in contrast to the ectothermic state, in the endothermic state the organism is more resistant to heat but relies less on HSPs as a first-line thermoprotective mechanism. Moreover, intraspecific, real-time, *in vivo* measurements in genetically diverse fowl strains relate improvement of thermoresistance in endotherms to improved body temperature (T_b) regulation, with a concomitant delay in the expression of HSPs. The time course of this delay and the T_b at which it occurs imply that the ontogenetic and evolutionary pathways leading to improved thermoresistance may have followed two, apparently non-related, parallel routes – cellular and peripheral (non-cellular). In search of other cellular components that differentially participate in the heat shock response, we revealed a significant expression of fatty acid synthase (FAS) in heat-exposed endotherms but not in ectotherms.

Key words: HSP, ectothermy, endothermy, thermotolerance, heat shock, *Gallus gallus domesticus*.

Introduction

Ectothermy and endothermy constitute the major thermoregulatory strategies in living organisms. While ectotherms can be prone to changes in body temperature (T_b) in correlation with ambient temperature (T_a) variations, the internal physiological milieu of endotherms remains relatively stable despite acute external thermal fluctuations. In this regard, Huey et al. (2002) have suggested that simple multicellular organisms might use biochemical mechanisms to cope with changes in temperature whereas complex multicellular organisms have alternative higher order mechanisms. The chicken (*Gallus gallus domesticus*) is ideal for comparing ectothermic and endothermic 'thermal states', as during ontogeny its embryo undergoes a transition from ectothermy to endothermy, which is completed, at the earliest, by hatching (day 21) (Hohtola and Visser, 1998).

Induced thermotolerance is generally referred to as the state at which whole organisms and cultured cells are transiently more resistant to killing by heat and other stressors, due to a short pretreatment at moderately elevated ambient temperatures. This thermotolerance is well correlated with the synthesis of heat shock proteins (HSPs; Parsell and Lindquist, 1994). Indeed, overexpression of HSPs extends life span and increases resistance to stresses in ectotherms (Morrow et al., 2004). HSPs are evolutionary conserved polypeptides that function as molecular chaperones to prevent and repair deleterious damages caused to proteins by environmental and physiological stresses (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993; Craig et al., 1994). The expression of HSPs is primarily regulated at the level of transcription by a family of heat shock transcription factors (HSFs) (Morimoto, 1998). Four members of the HSF gene family (HSF1–4) have been isolated and characterized in vertebrates, two of which, HSF1 and HSF3, act as stress-responsive transcriptional activators (Nakai, 1999). In the absence of stress, HSF1 and HSF3 are mostly located in the cytoplasm in an inactive state (Nakai et al., 1995; Wu, 1995; Morimoto, 1998). To induce transcriptional activity of heat shock genes, HSF1 and HSF3 must acquire DNA-binding activity, preceded by oligomerization to a trimeric state and nuclear localization (Tanabe et al., 1997). Compared with the mammalian HSF1, avian HSF1 has a lower potency of activating heat shock genes in cells subjected to heat stress (Inouye et al., 2003). It is becoming clearer, however, that, similar to the mammalian HSF1, avian HSF1 also possesses thermoprotective traits, independent of induction of heat shock genes (Nakai and Ishikawa, 2001; Inouye et al., 2003; Izu et al., 2004).

Different models have been suggested as to the identity of the cellular thermal sensor that triggers the HSP response. According to the classical model, the accumulation of denatured proteins in the cytoplasm triggers the synthesis of

HSPs (Ananthan et al., 1986). Other models raise the options of thermal sensation by the ribosome, at the level of translation (Van Bogelen and Neidhardt, 1990) or based on the autophosphorylative trait of the HSP70 family members (McCarty and Walker, 1991). Most tempting to adopt, however, is the suggestion that thermal sensation occurs at the membrane level (Vígh et al., 1998).

Our present study focuses on the relationship between the organism's thermal buffer capacity (i.e. ectothermy *vs* endothermy) and its heat shock response at the cellular and peripheral (non-cellular) levels. The peripheral level is the sum of physiological responses that involve feedback circuits that are involved in the control of body temperature.

We hypothesized that ectotherms and endotherms would differ in their heat shock responses as they possess different buffer capacities to deal with extreme ambient temperatures. We used ontogenetic development as an experimental model for the transition from ectothermy to endothermy and revealed the relationship between T_b regulation and the HSP response. We also compared, in real time, the *in vivo* heat shock response of genetically heat-resistant (desert strain), phenotypically heat-resistant (long-term induction of thermoresistance) and heat-sensitive fowls. We present a novel phenomenon of long-term induced thermotolerance. This embryonic induction of thermotolerance is expressed as increased HSP levels in the adult but does not confer improved survival upon heat stress. Finally, we metabolically labelled *in vivo* chicken embryos and postnatals (chicks) to reveal other cellular components that differentially participate in the heat shock response.

Materials and methods

Heat shock experiments in embryos and postnatals (chicks)

Three sets of embryonic heat shock experiments were carried out. The first two involved chicken strains that differ in their resistance to heat at maturity (Lohmann < Hy Line < Bedouin). In the first set, 12- and 18-day-old embryos (N=19, in two independent experiments) were exposed to 42°C or 43°C for 6 and 8 h or to 44°C for 6 h. In the second set, 18-day-old embryos (N=3, in two independent experiments) were exposed to 42°C or 43°C for 2–8 h. In the third set, 6-day-old Leghorn embryos (N=19) were exposed to 42°C for 6 h (see Long-term induction of thermotolerance).

Two sets of postnatal heat shock experiments were carried out. In the first set, 1-day-old postnatals (N=3, in two independent experiments) of three chicken strains that differ in resistance to heat at maturity (Lohmann<Hy Line<Bedouin) were exposed to 42°C or 43°C for 2–8 h. In the second set, 16-day-old Leghorn postnatals (N=24) were exposed to 24 h at 40°C (see Long-term improvement of survival – phenotypic thermoresistance). In each of the experiments, tissues were sampled for analyzing the expression of HSPs.

Protein extraction

For the purpose of protein extraction, dissected tissues (brain and liver) were homogenized in ice-cold buffer containing 0.1 mol l^{-1} NaCl, 20 mmol l^{-1} Tris pH 7.4, 0.2 mmol l^{-1} EDTA, 20% glycerol (v/v), 0.5 mmol l^{-1} dithiothreitol (DTT), 15 μg ml^{-1} leupeptin and 1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF). Samples were centrifuged for 30 min (4°C, 17 210 ***g***; Sorvall RC-5B; rotor ss-34), and supernatants were collected, frozen in liquid nitrogen and stored at –70°C. To extract protein from blood cells, blood samples were washed once with ice-cold phosphate-buffered saline and centrifuged for 2 min (4°C, 270 ***g***; Sorvall RC-5B; rotor ss-34). The cells were re-suspended in ice-cold TMP buffer (containing 10 mmol l^{-1} Tris pH 7.4, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} DTT, 15 μg ml^{-1} leupeptin and 1 mmol l^{-1} PMSF) and were frozen (liquid nitrogen) and thawed (37°C) in four cycles. Samples were centrifuged for 30 min (4°C, 17 210 ***g***; Sorvall RC-5B; rotor ss-34), and supernatants were collected, frozen in liquid nitrogen and stored at –70°C. Blood cell nuclear proteins were extracted as previously described by Dyer and Herzog (1995).

In vivo *metabolic labelling*

Protein labelling in chicken embryos was performed as described elsewhere (Banerji et al., 1987), with slight modifications. Briefly, three embryonated eggs (in two independent experiments) were transferred on day 18 of incubation from 37°C to 42°C or 43°C for up to 8 h. At 2 h intervals, embryos were directly injected subcutaneously with 200 μCi (7.4 MBq) of [^{35}S]methionine in 20 μl of PBS *via* a hole drilled through the shell, using a syringe equipped with a 25 G bent needle. The hole was then sealed with wax, and the eggs were incubated for 2.5 h at 37°C. Control embryos were maintained at 37°C. One-day-old postnatals (N=3, in two independent experiments) were transferred from 28°C to 42°C or 43°C for up to 8 h in a controlled climate chamber. At 2 h intervals, they were subcutaneously injected with 200 μCi (7.4 MBq) of [^{35}S]methionine in 20 μl of PBS, using a syringe adapted to a 25 G bent needle, then transferred back to 28°C for 2.5 h. Control postnatals were maintained at 28°C. Labelled tissues of embryos and postnatals were isolated, washed in PBS, homogenized, and the proteins separated by 10% acrylamide gels. Gels were subjected to fluorography and exposed for 4–16 h.

Long-term induction of thermotolerance

Long-term induction of thermotolerance was achieved by exposing 6-day-old Leghorn embryos (N=19) to a single thermal event, 6 h at 42°C [relative humidity (RH)=50±5%], within a temperature-controlled room (±0.3°C). We tested the induction of thermotolerance at maturity by exposing adult chickens (5 months of age) to 40°C for 8 h during two consecutive days.

Long-term improvement of survival – phenotypic thermoresistance

Improvement of survival at maturity (phenotypic

thermoresistance) was achieved by exposing 16-day-old Leghorn postnatals (N=24) to a single thermal event, 24 h at 40°C (RH=50±5%), within a temperature-controlled room (±0.3°C). Phenotypic thermoresistance was tested at maturity (5 months of age) by exposure for 8 h to 40°C during two consecutive days.

Real-time, in vivo *measurements of the heat shock response*

Three different groups of mature chickens (N=3 in each group) that differ in their resistance to heat were examined. The groups included individuals of the genetic heat-sensitive Leghorn strain, the phenotypic thermoresistant Leghorn strain (group 16d; see the previous section) and the genetic heat-resistant Bedouin strain. Twenty hours prior to each experiment, a polyethylene cannula (PE-50) was implanted in a wing vein of mature fowl under local anaesthesia (2% Lidocaine HCl), and a 5 cm-long, custom-made polyethylene cannula (PE-160) was implanted dorsal to the rectum and fastened to the skin. Experiments were carried out within a temperature-controlled room (±0.3°C). The birds had free access to food and water and could freely move in their individual cages. Each experiment started at 08.00–09.00 h at an ambient temperature of 24°C (RH=50±5%). A copper–constantan thermocouple was introduced into the rectal cannula, locked at a pre-determined depth of 5 cm and connected to a digital thermometer (±0.1°C). After ~10 min, T_b stabilized around 41°C, after which blood was remotely sampled through an extended PE-50 tubing. T_a was then elevated to 38°C (RH=50±5%). It took ~20 min to reach this temperature. Body temperature was monitored continuously and blood samples were taken at each 1°C increase in T_b up to 45°C (heating phase). At this time, T_a was lowered back to 24°C and blood samples taken at each 1°C decrease in T_b down to 41°C (recovery phase). After each blood sample, the cannula was flushed with heparinized saline.

Fatty acid synthase identification – mass spectrometry analysis

Protein extracts of brain and liver tissues of control and heat-exposed embryos (N=3) and postnatals (N=3) were run on a 7% acrylamide gel and stained with Coomassie blue. The stained protein bands, at a molecular mass of ~270 kDa, were cut from the gel with a clean razor blade and the proteins were reduced with 10 mmol l^{-1} DTT and modified with 100 mmol l^{-1} iodoacetamide in 10 mmol l^{-1} ammonium bicarbonate. The gel pieces were treated with 50% acetonitrile in 10 mmol l^{-1} ammonium bicarbonate to remove the stain, followed by drying the gel pieces. The dried gel pieces were rehydrated with 10% acetonitrile in 10 mmol l^{-1} ammonium bicarbonate containing 0.005 μg μl^{-1} trypsin, and then incubated overnight at 37°C. The resulting peptides were recovered with 60% acetonitrile with 0.1% trifluoroacetate. The tryptic peptides were resolved by reverse-phase high-performance liquid chromatography on 0.1×300-mm fused silica capillaries (J&W, Folsom, CA, USA; 100 μm i.d.) home-filled with porous R2 (Persepective, Framingham, MA, USA). The peptides were eluted using an 80-min linear gradient of 5–95% acetonitrile with 0.1% acetic acid in water at a flow rate of ~1 μl min^{-1}. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ; Finnigan, San Jose, CA, USA). Mass spectrometry was performed in the positive ion mode using repetitively full MS scan followed by collision induced dissociation (CID) of the most dominant ion selected from the first MS scan. The mass spectrometry data were compared to simulated proteolysis and CID of the proteins in the NR-NCBI database using the Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose, CA, USA). The amino terminal of the protein was sequenced on a Peptide Sequencer 494A [Perkin Elmer, (Applied Biosystems), Foster City, CA, USA] according to the manufacturer's instructions.

SDS–PAGE and western blot

Whole-cell and tissue lysates were boiled in sample application buffer containing 2-mercaptoethanol. Proteins were separated by SDS–polyacrylamide gel (10%) and transferred onto nitrocellulose membrane (Schleicher & Schuell Gmbh, Dassel, Germany). The membranes were probed with monoclonal anti-actin, anti-HSP70 (recognizing the constitutive and the inducible forms of the protein; Sigma H5147) and anti-HSP90 (Sigma H1775) antibodies or polyclonal anti-HSF1 and anti-HSF3 (a generous gift from Dr A. Nakai), followed by appropriate secondary antibodies. The proteins were visualized by enhanced chemiluminescence.

RNA isolation and northern blot

Total RNA was isolated from blood cells by TRI REAGENT-BD (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA (5 μg) was separated in formaldehyde–agarose gel and transferred onto nylon membrane (Zeta-Probe; Bio-Rad, Hercules, CA, USA). Chicken HSP70 cDNA (a kind gift from Dr R. Morimoto) was labelled by the extension priming method using [α-^{32}P]dATP. Membrane was hybridized for 16 h at 55°C, washed with 0.1% SDS in 1× SSC at 45°C, 50°C and 55°C and exposed to X-ray film (Kodak BioMax MS) at –70°C in the presence of an intensifying screen.

Electromobility shift assay (EMSA)

Electromobility shift assay was performed as previously described (Mosser et al., 1988). Briefly, equal amounts of cellular proteins (20 μg for brain extraction and 5 μg for blood nuclei proteins) were incubated with a ^{32}P-labelled double-stranded oligonucleotide (5′-CTAGAAGCTTCTAGAAGC-TTCTAG-3′). The protein-bound and free oligonucleotides were electrophoretically separated by 4% native polyacrylamide gels. The gels were dried and autoradiographed.

Statistical analysis

Differences between means (survival time of control and experimental groups in the case of long-term induction of

thermotolerance) as well as densitometry of bands were verified by unpaired *t*-test. The other parameters (hatchability of embryos from different strains) were subjected to Tukey's *post-hoc* analysis of variance (ANOVA). A value of $P<0.05$ was accepted as significant.

Results and discussion

Ectotherms but not endotherms may rely on HSPs for survival

The cellular heat shock response, often termed the HSP response, is characterized by induced synthesis of HSPs (Morimoto et al., 1990). The expression of HSPs is primarily regulated at the level of transcription by HSF (Morimoto, 1998). It is believed that increasing levels of HSPs ensure survival under stressful conditions. We addressed this general phenomenon by testing two possible thermoregulatory strategies (i.e. ectothermy *vs* endothermy). We approached this problem by looking at the development of embryos of three chicken strains that differ in resistance to heat at maturity (Lohmann < Hy Line < Bedouin), representing the ectothermic state, while the endothermic state was represented by postnatals (1–25 days post-hatching) of the same strains. Embryos and postnatals were exposed to common thermal stress conditions at 42°C or 43°C (the normal incubation temperature is 37–38°C), and their HSP response, hatchability, T_b and survival were measured. We identified an embryonic time window (between the ages of 12 and 18 days) that is the most heat-sensitive stage in the chicken's life cycle and during which their hatchability and heat shock response differed in relation to the severity of heat stress (Table 1). In general, up to 12 days of age, there was an apparent HSP response and hatchability was not affected by the heat stress (data not shown). Thereafter, embryos became more susceptible to heat stress. The heat shock response of all strains within this time window was characterized by an increased DNA-binding activity of HSF (Fig. 1A) and by increased levels of HSPs (unpaired *t*-test, $P<0.05$; Fig. 1B). A metabolic labelling analysis as a function of the duration of heat shock in 18-day-old embryos demonstrated that hatching success was strongly associated with higher levels of newly synthesized HSPs (Fig. 1C). The very similar pattern of protein synthesis in controls (37°C) and in embryos exposed to 42°C for 8 h suggests that the failure of the latter to hatch does not derive from a short-term heat-induced inhibition of general protein synthesis but rather from a block in the synthesis of HSPs that denies the rescue of the long-term effect of heat shock. All strains exhibited a similar HSP response (Fig. 1) and were similarly affected by heat stress, as shown by a significant decrease in their hatchability ($P<0.05$) within the above-described embryonic time window (Fig. 2). Thus, the superior heat resistance of the Bedouin fowl at maturity is not expressed in the embryonic stage. These findings imply that intraspecific variation in thermal resistance of mature endothermic fowl arises only after the transition from ectothermy to endothermy.

Remarkably, the peaks of thermosensitivity and thermoresistance during ontogeny were only 3–4 days apart; indeed, postnatals proved to be the most heat-resistant stage in the chicken's life cycle and, even after a 24 h exposure to 42–43°C, manifested only a small, though significant, increase in T_b, safely below the hyperthermal zone (data not shown). This slight increase in T_b was not accompanied by an HSP response. Nevertheless, the unique constitutive DNA-binding state of HSF (as seen in controls) was released at the end of the heat shock period (Fig. 3A). The heat shock response in avian cells is mediated by two transcription factors, HSF1 and HSF3, interacting with the heat shock consensus element (HSE) at the promoter of the heat shock genes (Nakai et al., 1995). The release of the HSE-bound HSF in the heat-shocked postnatals, however, did not result from decreased levels of either HSF [$P=0.11$ and $P=0.4$ (ns) for HSF1 and HSF3, respectively; Fig. 3B]. It has been suggested that, upon heat exposure, HSF also regulates the activity of non-heat shock genes (Westwood et al., 1991). Most striking, activated HSF1 negatively regulates the expression of febrile response mediators (Housby et al., 1999; Xiao et al., 1999). Logically, the products of such genes should be downregulated in heat-exposed organisms. We therefore suggest that part of the overall physiological mechanism of thermoregulation under elevated temperatures in postnatals is attained, at the cellular level, through a switch from positively regulating heat shock genes by HSF to a positive and negative mode of regulation of other genes. This switch may consequently explain the unchanged protein levels of HSP70 and HSP90 [$P=0.32$ and $P=0.25$ (ns), for HSP70 and HSP90, respectively] in response to heat exposure (Fig. 3C), which held for up to 25 days of age.

Table 1. *A summary of age-dependent responses of hatching and HSP expression to increasing thermal loads in 12- and 18-day-old Lohmann, Hy Line and Bedouin fowl embryos of different ages*

	T_a=42°C				T_a=43°C				T_a=44°C	
	Hatching*		HSP response†		Hatching*		HSP response†		Hatching*	HSP response†
Exposure length	6 h	8h	6 h	8 h	6 h	8 h	6 h	8 h	6 h	6 h
Embryonic age										
12 days	+	+	nr	nr	+	+	↑	↑	–	nr
18 days	+	–	↑	nr	+	–	↑	nr	–	nr

T_a, ambient temperature.
*+, hatching; –, reduced hatching.
†↑, increased response; nr, no response.

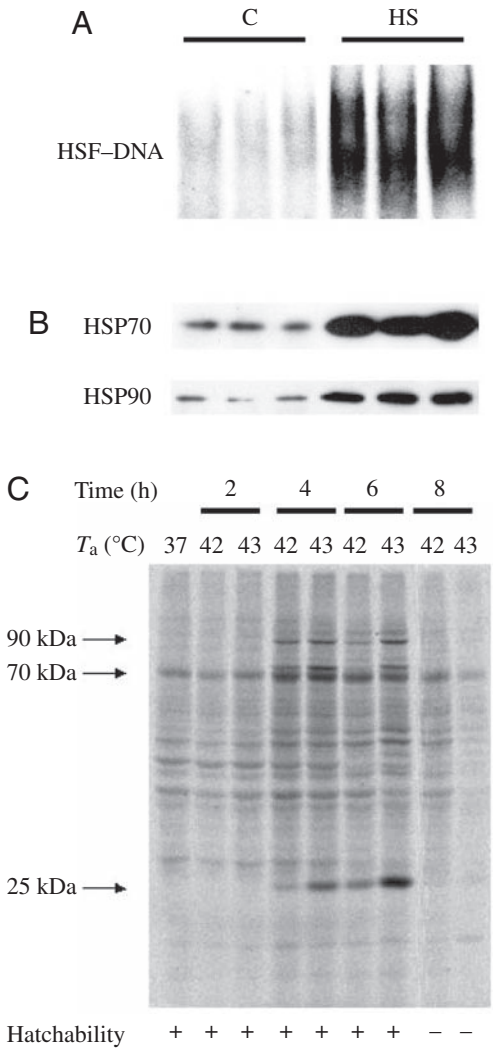

Fig. 1. A typical embryonic (18 days, brain tissue) HSP response (representing Lohmann, Hy Line and Bedouin fowl strains) under heat shock conditions (6 h at 42°C) that precede the deleterious effects on hatchability (N=6 in each treatment). (A) HSF–DNA-binding activity detected by EMSA. EMSA was performed with a [^{32}P]HSE oligonucleotide and whole brain tissue extracts. (B) Western blot analysis of HSP70 and HSP90. Each lane represents a different individual. C, control, unexposed individuals; HS, at the end of the heat exposure. A similar pattern was observed in 12-day-old embryos exposed to 43°C. (C) *In vivo* metabolic labelling analyzed by [^{35}S]methionine incorporation. T_a, ambient temperature; time (h), heat shock period in hours.

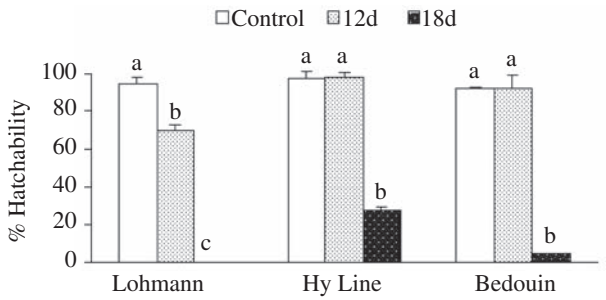

Fig. 2. The effect of embryonic heat exposure (8 h at 43°C) on hatchability in three layer strains (N=19) of fowl that differ in their heat resistance at maturity (Lohmann < Hy Line < Bedouin). Control, maintained at 37°C for the entire incubation period; 12d, heat shocked on day 12 of incubation; 18d, heat shocked on day 18 of incubation. Hatchability was markedly reduced in all strains on day 18. Different letters within each strain denote significant differences (ANOVA, P<0.05). Eggs of the Lohmann strain did not hatch at all if heat shocked on day 18.

Metabolic labelling of 1-day-old postnatals confirmed that there was no net increase of newly synthesized HSPs during 8 h of heat exposure (Fig. 3D). Therefore, these results strongly support our suggestion that, under identical heat shock conditions, the HSP system in postnatals, in contrast to embryos, may play only a minor role in thermoprotection.

Acquired thermotolerance and survival in endotherms are not linked

Adaptation to elevated ambient temperatures may be divided into thermotolerance and heat acclimatization. Whereas thermotolerance refers to cellular adaptation *via* a prerequisite accumulation of HSPs, acclimatization is determined by the organism's ability to maintain thermal equilibrium in the heat (Moseley, 1997). Acquired thermotolerance has been implicated in increased resistance to killing caused by extreme heat exposure in many ectotherms and in cell cultures of endothermic organisms (Feder and Hofmann, 1999). Here, we address the relationship between HSP accumulation and survival in endotherms, at the whole organism level. We approached this issue by a long-term induction of thermotolerance through embryonic conditioning and by a long-term improvement of survival through postnatal conditioning. Our findings show that embryonically conditioned individuals can acquire thermotolerance at maturity, as expressed by improvement of their HSP response (unpaired t-test, P<0.05; Fig. 4). Since survival time did not differ significantly among control and embryonically conditioned groups (unpaired t-test, P=0.34; Fig. 4, bottom), this acquired thermotolerance is not correlated with improvement of survival. Alternatively, survival of mature individuals from a heat-sensitive strain was significantly improved as a result of a single postnatal heat exposure (16-day-old Leghorn group). This type of 'phenotypic adaptation' (acclimatization) is characterized by a delayed HSP response (Fig. 5B–D) and is distinct from the typical acquired thermotolerance because of its different time course and duration (more than 5 months, compared with a few hours or days for typical acquired thermotolerance). Altogether, these findings indicate that, at least in endothermic birds, at the whole organism level, survival does not depend on a prerequisite accumulation of HSPs.

Thermoresistance in endotherms is characterized by improvement of T_b *regulation and by a delayed HSP response*

How is the heat shock response reflected in various states of

thermoresistance in endothermy, and what accounts for improved thermoresistance of endotherms if not HSPs? To gain insight into these issues, we monitored in real time the *in vivo* heat shock response within the same individuals of mature fowl of various thermal histories: control Leghorns (heat sensitive), 16-day-old Leghorns (phenotypically adapted) and the Bedouin fowl (genetically resistant). Within the Leghorn line, the two groups represent identical genotypes but different states of phenotypic thermoresistance. The intraspecific variation (Leghorn *vs* Bedouin) represents a genetic difference. Our previous comparisons of the commercial egg-layer Leghorn chicken and the desert-origin, genetically heat-resistant Bedouin fowl identified various thermoregulatory mechanisms that contribute to the superiority of the Bedouin fowl with respect to heat resistance (Arad, 1983; Arad and Marder, 1982; Marder et al., 1974). Thus, we hypothesized that the cellular heat shock response would also differ among non-resistant, phenotypically and genetically heat-resistant fowl. A distinct pattern of T_b regulation and HSP response was revealed for each group. In general, the Bedouin fowl was superior to the heat-sensitive Leghorn in its lower heating rate and in its higher cooling rate (Fig. 5A) and was characterized by a considerably delayed HSP response, both in relation to time scale and to T_b level (Fig. 5B–D). The 16-day-old Leghorn group revealed intermediate, significantly different patterns (Fig. 5A–D). These novel findings are of evolutionary significance, since they suggest that fowl's HSP responses, unlike in ectotherms (Krebs and Feder, 1997; Michalak et al., 2001; Ul'masov et al., 1992), do not contribute to the genetic variations of heat shock tolerance, but rather it is the effectiveness of the homeostatic mechanisms for T_b regulation.

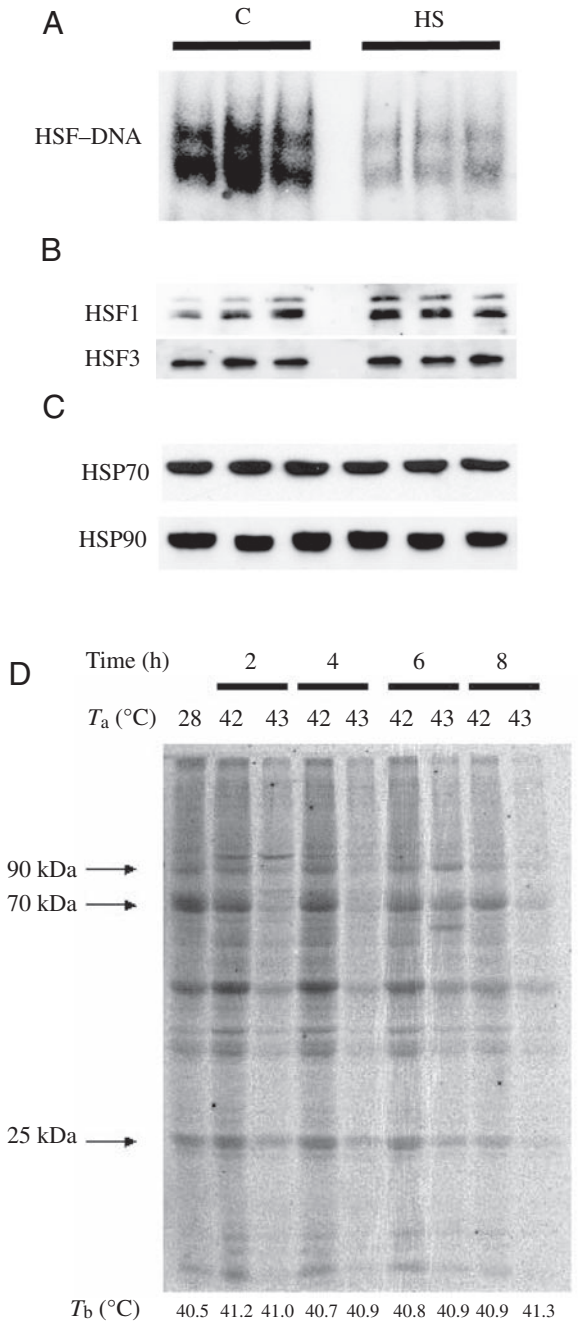

Fig. 3. A typical HSP response of postnatals (1 day, brain tissue) exposed to 6 h or 24 h at 42°C (N=6 in each treatment). (A) HSF–DNA-binding activity detected by EMSA. EMSA was performed with a [^{32}P]HSE oligonucleotide and whole brain tissue extracts. Western blot analysis of HSF1 and HSF3 (B) or HSP70 and HSP90 (C). Each lane represents a different individual. C, control, unexposed individuals; HS, at the end of the heat exposure. A similar pattern was observed up to 25 days of age. (D) *In vivo* metabolic labelling analyzed by [^{35}S]methionine incorporation. T_a, ambient temperature; T_b, body temperature.

Fatty acid synthase is upregulated in postnatals in response to heat shock

In search of a cellular component other than HSPs that may participate in the thermoprotective process in endotherms, we

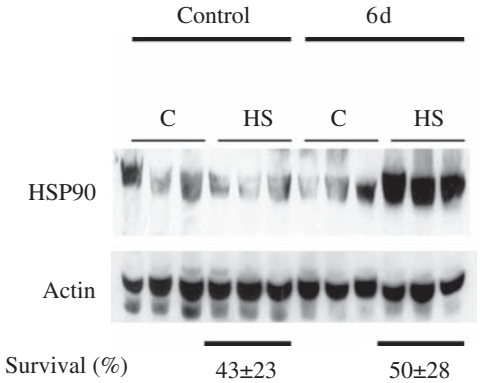

Fig. 4. Acquired thermotolerance of mature Leghorn individuals following a single embryonic heat exposure. Presented is the expression of HSP90 in the brain tissue. Actin levels were used to detect equal amounts of loaded proteins. Control, not exposed to heat as embryos; 6d, exposed to heat (6 h at 42°C) on day 6 of incubation; C, unexposed mature individuals (5 months of age); HS, heat exposure at maturity (8 h at 40°C during two consecutive days). Percentage survival (at the bottom of the figure) was not affected by the embryonic heat exposure (unpaired t-test, P=0.34) and is expressed as means ± S.D. (N=19).

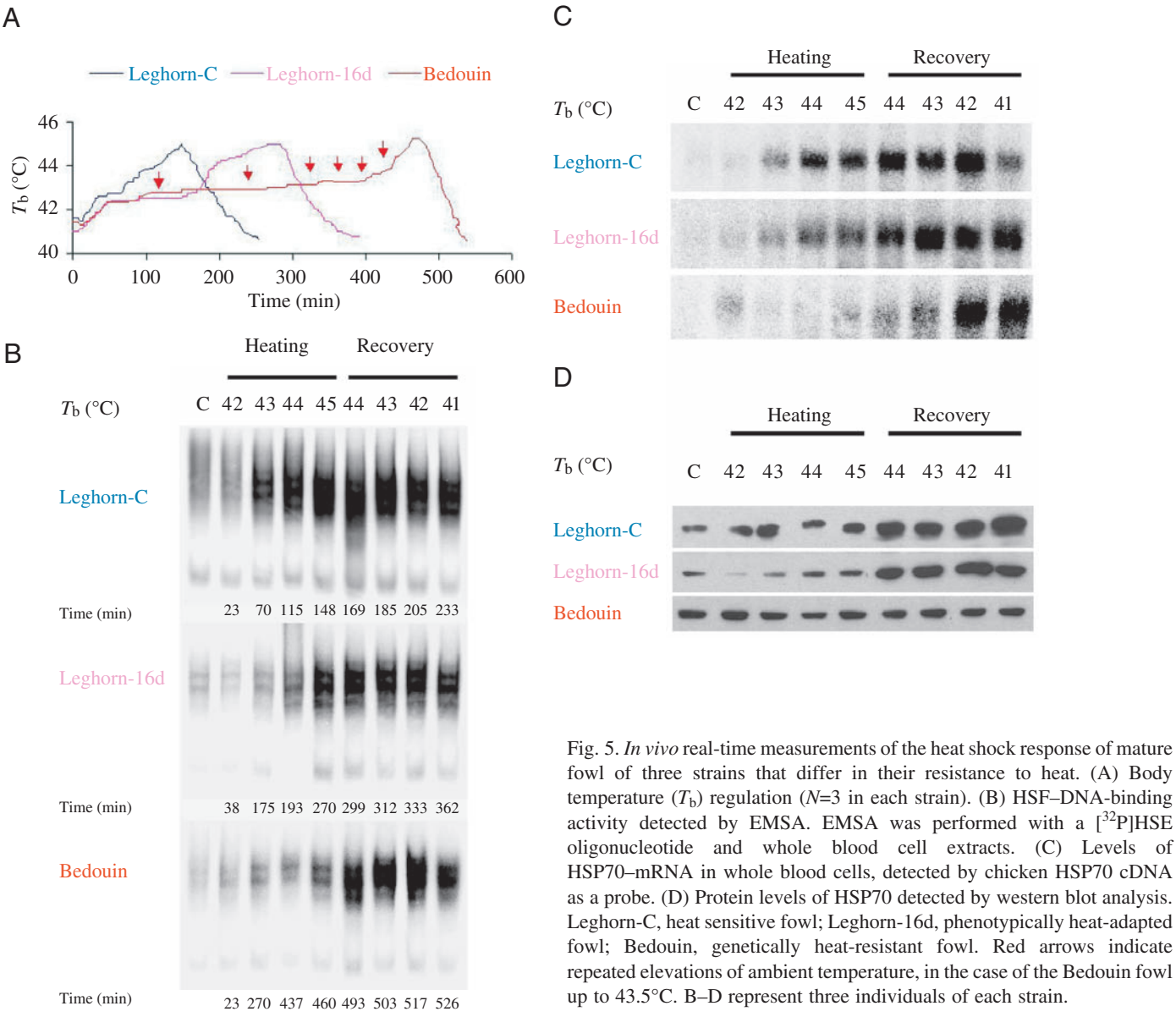

Fig. 5. *In vivo* real-time measurements of the heat shock response of mature fowl of three strains that differ in their resistance to heat. (A) Body temperature (T_b) regulation (N=3 in each strain). (B) HSF–DNA-binding activity detected by EMSA. EMSA was performed with a [^{32}P]HSE oligonucleotide and whole blood cell extracts. (C) Levels of HSP70–mRNA in whole blood cells, detected by chicken HSP70 cDNA as a probe. (D) Protein levels of HSP70 detected by western blot analysis. Leghorn-C, heat sensitive fowl; Leghorn-16d, phenotypically heat-adapted fowl; Bedouin, genetically heat-resistant fowl. Red arrows indicate repeated elevations of ambient temperature, in the case of the Bedouin fowl up to 43.5°C. B–D represent three individuals of each strain.

performed *in vivo* metabolic labelling followed by mass spectrometry analysis of heat-shocked embryos and postnatals. A dramatic increase (P<0.05) in the expression of fatty acid synthase (FAS) was revealed in postnatals, but not in embryos, in response to heat shock (Fig. 6). Abrupt elevation of temperature affects the cell membrane physical state by increasing its fluidity (Dynlacht and Fox, 1992; Mejia et al., 1995; Vígh et al., 1998). However, cells may compensate for thermal disturbances through physiological and biochemical mechanisms that allow them to maintain homeostatic equilibrium. One such mechanism, termed homeoviscous adaptation, allows cells to regulate membrane fluidity by adjustment of its lipid composition (Carratù et al., 1996; Vígh et al., 1998). Upon exposure to low temperature, a reduction in the membrane fluidity triggers an increase in the expression of desA, a desaturase that subsequently leads to the desaturation of membrane lipids (Vígh et al., 1993). At high temperature, it has been demonstrated that HSP17 transcription was strongly regulated by subtle changes in membrane physical order (Horváth et al., 1998; Lee et al., 2000). HSP17 has been further shown to have a dual role as a ‘membrane stabilizing factor’ and as a member of a multi-chaperon protein folding network (Török et al., 2001). However, no causal link has been proposed, upon exposure to elevated temperature, between compensative factors regulating membrane fluidity, cellular thermoprotection and the expression of HSPs. The current study suggests that FAS may play a role in this regulation as it catalyses the synthesis of saturated long-chain fatty acids from acetyl CoA, malonyl CoA and NADPH. Based on our findings, and supported by the findings of Horváth et al. (1998), who reported a causal relationship between the membrane physical state and the threshold temperature for activation of heat shock genes, we suggest a possible role for FAS in modulating the cellular heat shock response in

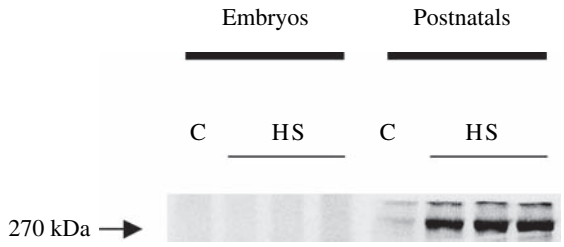

Fig. 6. *De novo* synthesis of a 270 kDa protein in liver tissue of 1-day-old postnatals in response to heat shock (2 h, 42°C). No such response was observed in 18-day-old embryos (score zero in densitometry of both control and heat-shocked individuals). This protein was identified by mass spectrometry analysis as fatty acid synthase (FAS). Each lane represents a different individual. C, control, unexposed individuals; HS, at the end of the heat exposure.

endotherms. Accordingly, increasing levels of this enzyme in response to elevated temperatures may contribute to rigidifying the membrane and thereby raising the threshold temperature for synthesis of HSPs. This suggestion may serve as a foundation for future studies.

Conclusions

Our study shows that, despite the likely contribution of HSPs to the expansion of the cellular thermal safety margins in endothermic birds, HSPs do not improve their thermal resistance and thus do not constitute a first-line thermal defense mechanism. The ectothermic state, however, is strikingly different. Here, the increased levels of HSPs precede and buffer the deleterious effect of heat on embryo hatchability, suggesting a tight correlation between HSPs and survival. This ectothermy-to-endothermy transition concept is supported by findings that ectothermic species that live in widely fluctuating thermal habitats possess a stronger HSP protective trait compared with ectotherms that inhabit stable thermal niches and lack or have a weaker HSP response (Bosch et al., 1988; Hofmann et al., 2000; Sanders et al., 1991).

The distinct evolutionary adaptations of the Bedouin fowl (Arad, 1983; Arad and Marder, 1982; Marder et al., 1974) were clearly revealed in the kinetics of the heat shock response in the present study, even after 9 years of maintenance in captivity under non-desert conditions. At the cellular level, these genetically inherited traits could result from the establishment of different thresholds for induction of HSPs: slightly different biochemical properties of HSF (Feder and Hofmann, 1999), different cellular environments (Clos et al., 1993), autoregulatory processes (Morimoto, 1998), variations in thermal stability of cellular proteins (Somero, 1995) and different membrane characteristics (Vígh et al., 1998; present study). At the peripheral level, the different time course of the heat shock response (Fig. 5A) could reflect distinct capacities of the homeostatic mechanisms (Arad, 1983).

Based on our findings, such intraspecific variations are likely to occur also upon ontogenetic transition from ectothermy to endothermy. We thus suggest that the ontogenetic and the intraspecific evolutionary pathways of thermoresistance in fowl may have followed two, apparently non-related, parallel routes: first, a cellular route, in which the acquisition of thermoresistance is not HSP-dependent and could result from altered mechanisms of thermal sensation; second, a peripheral route, characterized by altered homeostatic mechanisms that lead to differential patterns of T_b regulation.

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