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Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae

Scott A. L. Hayward*, Joseph P. Rinehart and David L. Denlinger
Ohio State University, Department of Entomology, 318 W. 12th Ave, Columbus, OH 43210, USA
*Author for correspondence (e-mail: hayward.23@osu.edu)

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Summary

Heat shock proteins (Hsps) are a ubiquitous component of the cellular response to stress in both prokaryotic and eukaryotic organisms, but their role and function during desiccation stress in terrestrial arthropods has received limited attention. Molecular responses to rehydration are arguably as important as those to desiccation in maintaining cellular integrity and enzyme activity, but the role of Hsps during stress recovery is poorly understood and has never been addressed with respect to rehydration in insects. This study identifies distinct differences in the Hsp response to desiccation and rehydration in the flesh fly *Sarcophaga crassipalpis*, as well as differences in the desiccation responses of diapausing and nondiapausing pupae. In nondiapausing pupae, the expression of two inducible Hsps (Hsp23 and Hsp70) is upregulated by desiccation, but the water loss threshold for Hsp expression changes at different rates of dehydration. Continued desiccation results in the prolonged expression of both Hsp23 and Hsp70, which may contribute to the delayed adult eclosion noted in samples desiccated for more than 3 days at <5% relative humidity/25°C. In diapausing pupae, *hsp23* and *hsp70* transcripts are already highly expressed and are not further upregulated by desiccation stress. Both of the constitutive Hsps investigated, Hsp90 and Hsc70, were unresponsive to desiccation in both nondiapausing and diapausing pupae. However, both Hsp90 and Hsc70 were upregulated upon rehydration in nondiapausing and diapausing pupae. These results indicate distinct roles for the different Hsps during desiccation stress and rehydration/stress recovery. The response to desiccation recovery (rehydration) is similar to the Hsp response to cold recovery identified in *S. crassipalpis*: Hsp90 and Hsc70 are upregulated in both cases.

Key words: diapause, insect, *Sarcophaga crassipalpis*, stress, water loss, heat shock protein, flesh fly, desiccation, rehydration.

Introduction

Few variables have a greater influence on the distribution and abundance of terrestrial organisms than moisture availability. Terrestrial arthropods in particular, due to their high surface area to body volume ratio, require solutions to problems associated with water balance (Hadley, 1994), and numerous contributing physiological mechanisms have been investigated: for example, osmolyte synthesis (Bayley and Holmstrup, 1999), membrane adaptation (Holmstrup et al., 2002), volume regulation (Zachariassen and Pedersen, 2002), water conservation (Gibbs et al., 2003) and osmoregulation (Sømme, 1994; Naidu, 2001). By contrast, the function and regulation of proteins during insect dehydration (and associated changes in gene expression) have received limited attention, despite the fact that proteins are an important component of the desiccation stress response in other organisms (Potts, 1994; Ingram and Bartels, 1996; Liang and MacRae, 1999; Browne et al., 2002).

Among insects, dehydration is known to induce the expression of a novel desiccation protein (dsp28) in the beetle *Tenebrio molitor* (Graham et al., 1996) and to increase the abundance of heat shock protein (Hsp) transcripts in several species (Tammariello et al., 1999; Bayley et al., 2001). Hsps are well known for their role as molecular chaperones and they function in the cellular stress response in organisms as diverse as bacteria, yeast, plants and humans (Feder and Hofmann, 1999; Fink, 1999). Based on their molecular mass, three size categories of Hsps are well documented in insects: small Hsps (~20–30 kDa), the Hsp70 group (~70 kDa) and the 90 kDa group. Representatives of each group have been cloned and sequenced from the flesh fly *Sarcophaga crassipalpis*, and patterns of expression have been monitored in response to a variety of environmental insults (Yocum et al., 1998; Rinehart and Denlinger, 2000; Rinehart et al., 2000). Desiccation stress increases the abundance of *hsp23* and *hsp70* transcripts in nondiapausing pupae of this species (Tammariello et al., 1999), but little more is known about this response. What other Hsps are responsive to dehydration in *S. crassipalpis*? At what level of water loss is Hsp transcription initiated? Do transcription thresholds differ between Hsps or between different rates of water loss?

The desiccation response during diapause is also of considerable interest because pupae in diapause have no access to free water for long periods of time (Yoder and Denlinger, 1991) and certain Hsps are developmentally upregulated throughout this period (Yocum et al., 1998; Rinehart et al., 2000). Increased Hsp expression during diapause has also been reported in several other invertebrates (Liang and MacRae, 1999; Yocum, 2001; Denlinger, 2002). In *S. crassipalpis*, *hsp23* and *hsp70* transcripts are maximally expressed during diapause, even in the absence of stress (Yocum et al., 1998; Rinehart et al., 2000). Neither heat (45°C) nor cold (–10°C) treatment further enhances this response. Constitutively expressed Hsp90 is downregulated during diapause yet remains responsive to both heat and cold stress (Rinehart and Denlinger, 2000). Another constitutive Hsp, heat shock cognate 70 (Hsc70), is unchanged by diapause and seems more responsive to cold than heat stress (Rinehart et al., 2000). The Hsp response to desiccation during diapause in *S. crassipalpis* has not yet been examined. Hsc70 may be particularly interesting in this respect as adaptations to cold and desiccation often have overlapping characteristics (Ring and Danks, 1994; Block, 1996).

In addition to the stress caused by desiccation, the rapid uptake of water during rehydration has the potential to elicit cell damage. Consequently, physiological and molecular responses to rehydration are arguably as important as those to desiccation. In the moss *Tortula ruralis*, most of the molecular repair mechanisms are thought to be initiated during rehydration rather than desiccation (Oliver, 1991; Bewley and Oliver, 1992). Rehydration-specific gene expression has also been reported in higher plants, in conjunction with the decline of dehydration-specific gene products (Bernacchia et al., 1996). Although Hsps have not been identified as key components of the rehydration response in plants, their known chaperone function suggests a potentially important role during the re-initiation of ‘normal’ protein synthesis upon removal of stress. Certain Hsps do indeed appear to function during stress recovery (Van Nieuwenhoven et al., 2001), including recovery from cold shock in *S. crassipalpis* (Rinehart et al., 2000). Thus far, molecular responses to rehydration in insects have not been examined.

To determine how Hsps influence stress tolerance at the organismal level, it is useful to characterize the responses of different Hsps across stress gradients. Desiccation studies are particularly instructive in this respect, as they provide the opportunity to quantify both the level of stress imposed (e.g. relative humidity) and the level of stress experienced (e.g. the amount and/or rate of water loss), which is not possible with thermal stress. Desiccation stress was utilized in this study to identify differences in the Hsp response of nondiapausing and diapausing pupae of the flesh fly *S. crassipalpis*, as well as changes in Hsp transcription thresholds in relation to different rates of water loss. We report distinct differences in the Hsp response to desiccation and rehydration in this species that suggest different functions for the constitutive and inducible groups of Hsps.

Materials and methods

Insect rearing

All experimental flies were from a laboratory colony of the flesh fly *Sarcophaga crassipalpis* Macquart. Nondiapausing individuals were reared throughout their life cycle under long-day conditions (15 h:9 h light:dark) at 25°C. Pupal diapause was induced by exposing adult flies to short daylengths (12 h:12 h L:D) and 25°C until larviposition, with larvae and pupae then maintained at 12 h:12 h L:D and 20°C (Denlinger, 1972).

Desiccation treatments

Three days after pupariation, nondiapausing pupae were transferred to constant 75% relative humidity (RH) conditions, maintained using saturated NaCl solutions, at 25°C for 24 h to synchronise their hydrated state prior to desiccation. The desiccation assessment was conducted with both intact day 4 nondiapausing pupae and samples from which the operculum of the puparium (anterior cap) had been removed. Samples were placed inside 2 ml centrifuge tubes (N=5 per tube) perforated with 20×1 mm holes, which in turn were placed in 15 ml containers filled with anhydrous calcium sulphate (Drierite®) and maintained at <5% RH/25°C. Samples were removed at 24 h intervals over a 7 day period. The following characteristics were assessed:

Survival and emergence time

Only individuals completely extricated from the puparium were considered survivors. Emergence time was recorded as the number of days between pupariation and emergence. Eclosion patterns within the long-day L:D cycle were also noted. Emergence was monitored at 25°C by placing pupae within an eclosion counting device (Yocum et al., 1994) set to record eclosion events every 2 h. RH conditions during this period were 59.5±1.4% RH (mean ± S.E.M.; N=20).

Water loss

Five pupae were selected for monitoring daily water loss. Individuals were weighed prior to desiccation (fresh mass) and upon removal from the Drierite® (desiccated mass) at each 24 h interval. Samples were then dried to constant mass at 50°C and their dry mass was noted. From these values, mean initial water content and percentage water loss were calculated. Four replicates were conducted; thus, for each value, N=20. Linear regression analysis determined the relationship between percentage water loss (arcsine transformed) and time at <5% RH/25°C. Differences between regression coefficients were calculated following Fowler and Cohen (1990).

hsp expression

Total RNA was extracted by homogenizing samples in TRIzol® reagent using standard protocol. Only live animals, i.e. those showing no tissue discoloration, were used. RNA from three animals was pooled for each sample, and hsp expression was assessed using northern blot hybridization.

Northern blot hybridization

Total RNA from each sample (20 μg) was loaded on a 1.5% agarose, 0.41 mol l^{-1} formaldehyde gel for electrophoresis. Samples were transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by downward capillary action using alkaline transfer buffer (Schleicher and Schuell, Inc., Keene, NH, USA). Northern blot hybridization was performed following Sambrook et al. (1989), using the following *S. crassipalpis* clones as templates to make DNA probes: Hsp23 (GenBank accession no. U96099; Yocum et al., 1998), Hsp70 and Hsc70 (GenBank accession no. AF107338 and AF107339, respectively; Rinehart et al., 2000) and Hsp90 (GenBank accession no. AF261773; Rinehart and Denlinger, 2000). 28s ribosomal RNA was used as a control gene. Each probe was labeled with digoxigenin-11dUTP using DIG High Prime (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization, washing and detection were undertaken following the DIG High Prime labeling and detection standard protocol. Membranes were exposed to X-ray film (Fuji) for 25–30 min at room temperature.

Diapause samples

Day 10 diapausing pupae were transferred to 25°C (75% RH) and allowed to acclimatize for 5 days (day 15 of diapause). The operculum of the puparium was removed from all day 15 diapause samples, which were then desiccated at <5% RH/25°C as described for nondiapausing pupae. Samples were removed at 3 day intervals over a 15 day period to assess survival, water loss and hsp expression as previously described. This procedure was repeated with day 30 diapausing pupae. Thus, day 15 diapause samples were desiccated from day 15 to day 30 of diapause, and day 30 diapause samples were desiccated from day 30 to day 45 of diapause.

Diapause termination under desiccation stress

hsp transcript expression was also monitored following diapause termination, elicited by hexane application (Denlinger et al., 1980), while individuals were under desiccation stress. Day 15 diapausing pupae (with operculum removed) were placed under <5% RH/25°C conditions, and after 9 days of desiccation the first RNA sample was collected. The remaining pupae were treated with hexane to terminate diapause under desiccating conditions by applying 5 μl of hexane to the head of each pupa. RNA samples were collected 3, 6, 9, 12 and 24 h post diapause termination, and hsp expression was noted as previously described.

Rehydration experiments

Day 4 nondiapausing pupae (operculum removed) were desiccated for 4 days at <5% RH/25°C and then transferred to either 75% or 100% RH conditions. After 24 h and 48 h of rehydration, total RNA was extracted and hsp expression assessed. Ten pupae were randomly selected to represent each condition and their mass monitored as previously described to determine initial water content, water loss and water gain through rehydration. This procedure was repeated with day 15 diapausing pupae (operculum removed).

Results

Water loss, survival and eclosion patterns following desiccation

To first document the intensity of the desiccation stress experienced during each dehydration experiment, we monitored water loss, survival and adult eclosion patterns in flies that were stressed as either nondiapausing or diapausing pupae. The mean mass of nondiapausing pupae was 112.7±5.7 mg (mean ± S.E.M.; *N*=120), with a mean body water content of 68.6±0.3% (mean ± S.E.M.; *N*=120). Water loss was more rapid if the operculum of the puparium (anterior cap) was removed than if the puparium remained intact (Fig. 1A). When the puparium was open, pupae lost almost 20% of their initial water content after 5 days at <5% RH/25°C. The relationship between water loss and time at <5% RH was significant for both intact and open puparia (Table 1), but the difference between regression coefficients was not significant (t=0.668, d.f.=12). Survival of nondiapausing flies with an open puparia declined throughout the desiccation period (Fig. 1A), with no individuals surviving more than 6 days at <5% RH/25°C. Survival of flies with an intact puparia also declined but never dropped below a mean value of 80% (Fig. 1A). The mean time interval from pupariation to adult eclosion for flies in an intact puparium was 12 days at 25°C and remained the same for all durations of desiccation that were tested (Fig. 1A). If the puparium was opened, the mean time from pupariation to adult emergence was 12 days for samples desiccated for 1 or 2 days but increased to a maximum of 16 days with 4–6 days of desiccation (Fig. 1A). Desiccation stress did not alter the circadian pattern of early dawn emergence noted in controls (data not shown).

The mean mass of diapausing pupae at the outset of the experiment was 110.8±1.7 mg (mean ± S.E.M.; *N*=120), with a mean water content of 67.0±0.2% (mean ± S.E.M.; *N*=120). Water loss increased throughout the 15 day desiccation period for diapausing pupae of both ages (Fig. 1B). The relationship between water loss and duration of desiccation was significant for both diapause groups (Table 1) but did not differ significantly between day 15 and day 30 samples

Table 1. *Linear regression of mean % water loss (arcsine transformed) and time spent at <5% RH/25°C for nondiapausing (ND) and diapausing (D) pupae*

	N	r^2	Regression coefficient	*F*	*P*
ND pupae					
Puparium opened	8	0.956	0.048	130.9	<0.001
Puparium intact	8	0.984	0.021	424.8	<0.001
D pupae					
Day 15	6	0.923	0.008	47.8	0.002
Day 30	6	0.96	0.012	96.8	<0.001

(t=0.18, d.f.=10). Survival of both day 15 and day 30 diapausing pupae declined at a similar rate as a result of desiccation (Fig. 1B).

hsp transcript expression

In nondiapausing flies, transcripts of *hsp23* and *hsp70* became abundant in response to desiccation after 2 days at <5% RH (Fig. 2A) in pupae encased in an opened puparium. Transcript abundance remained elevated throughout the desiccation treatment at a level equivalent to that resulting from heat shock. The upregulation of hsp23 and hsp70 was not noted in pupae encased in an intact puparium (Fig. 2B). Expression of *hsc70* and *hsp90* transcripts was unaltered by this level of desiccation stress in pupae encased in either open or intact puparia (Fig. 2).

Transcript levels of all Hsps investigated remained unresponsive to desiccation stress in day 15 diapausing pupae (Fig. 3A). In day 30 diapause samples, the expression of *hsp70* and *hsp23* transcripts remained high until day 6 of desiccation, disappeared on day 9 and day 12 but reappeared on day 15 at <5% RH (Fig. 3B). *hsp90* transcripts were upregulated on day 12 and day 15 of desiccation in these day 30 pupae, while levels of *hsc70* transcripts remained constant. The conspicuous drop in expression of hsp23 and hsp70 after day 6 of desiccation in day 30 diapause samples coincided with diapause termination in these pupae. The upregulation of hsp90 on day 12 and day 15, i.e. day 42 and day 45 pupae, is also consistent with diapause termination (Rinehart and Denlinger, 2000). The renewed expression of hsp23 and hsp70 on day 15 of desiccation suggests that, once development has been reinitiated, the fly again responds to desiccation stress by expressing these Hsps.

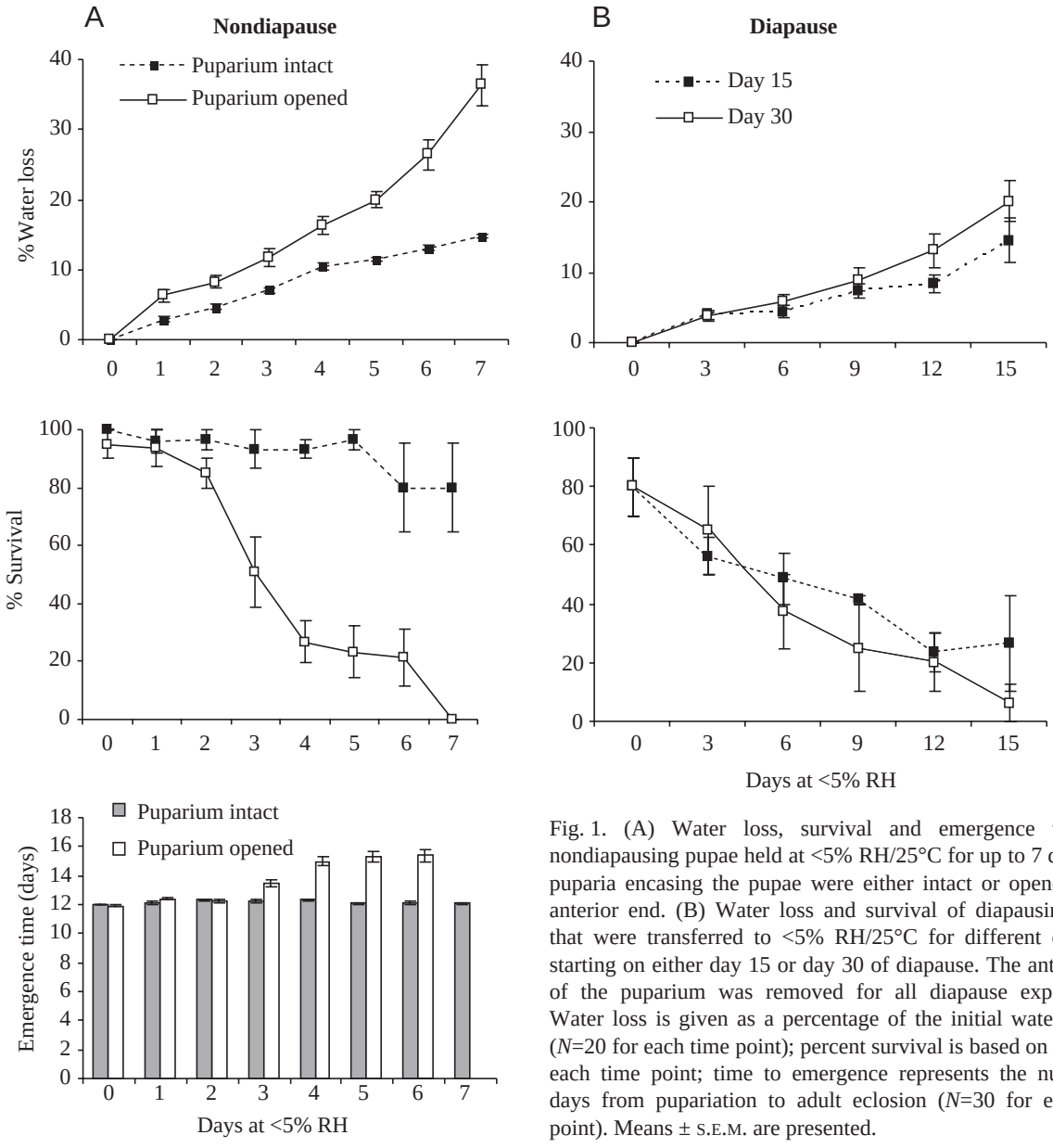

Fig. 1. (A) Water loss, survival and emergence time for nondiapausing pupae held at <5% RH/25°C for up to 7 days. The puparia encasing the pupae were either intact or opened at the anterior end. (B) Water loss and survival of diapausing pupae that were transferred to <5% RH/25°C for different durations starting on either day 15 or day 30 of diapause. The anterior cap of the puparium was removed for all diapause experiments. Water loss is given as a percentage of the initial water content (N=20 for each time point); percent survival is based on N=30 for each time point; time to emergence represents the number of days from pupariation to adult eclosion (N=30 for each time point). Means ± S.E.M. are presented.

Diapause termination under desiccation stress

The association noted above between the onset of development and the expression of Hsps under desiccation stress prompted a second approach to testing our hypothesis that Hsp23 and Hsp70 must be turned off to initiate development, even under stressful conditions. This was achieved by using hexane, as described by Denlinger et al. (1980), which promptly terminates diapause without causing mortality. In response to hexane, diapausing pupae break diapause immediately; within 12 h (20°C), the transcript abundance of Hsps that have been upregulated during diapause (Hsp23 and Hsp70) is undetectable (Yocum et al., 1998; Rinehart et al., 2000), and Hsp90, an Hsp that is downregulated during diapause, is upregulated. When this hexane tool was applied to terminate diapause under desiccation stress, the abundance of both *hsp23* and *hsp70* transcripts declined within 3 h of hexane application (Fig. 4). After 6 h, *hsp23* and *hsp70* transcripts were highly expressed again, but after 9 h were greatly diminished (*hsp23*) or were undetectable (*hsp70*). Consistent with earlier observations, *hsp90* transcript abundance increased 6 h after hexane application. These results thus imply that in order to initiate adult development both Hsp23 and Hsp70 must be downregulated, even under severe desiccation stress.

Water content and survival following rehydration

As anticipated from an earlier study on *S. crassipalpis* (Yoder and Denlinger, 1991), pupae were capable of absorbing atmospheric water vapor. One day of rehydration at either constant 75% or 100% RH returned the water content of nondiapausing pupae to values recorded prior to desiccation and, in the case of diapausing pupae, the rehydrated water content actually exceeded the initial predesiccated level (Fig. 5). Survival of both nondiapausing and diapausing pupae increased as a result of rehydration and reached levels similar to the survival noted in nondesiccated controls (Fig. 5).

hsp transcript expression in response to rehydration

hsp23 and *hsp70* transcripts were undetectable during rehydration in nondiapausing pupae until 2 days at 100% RH (Fig. 6A). In diapausing pupae, both hsp23 and hsp70 continued to be expressed during rehydration (Fig. 6B). In other words, the expression patterns were the same as in the nondesiccated controls reared under these two different developmental programs. By

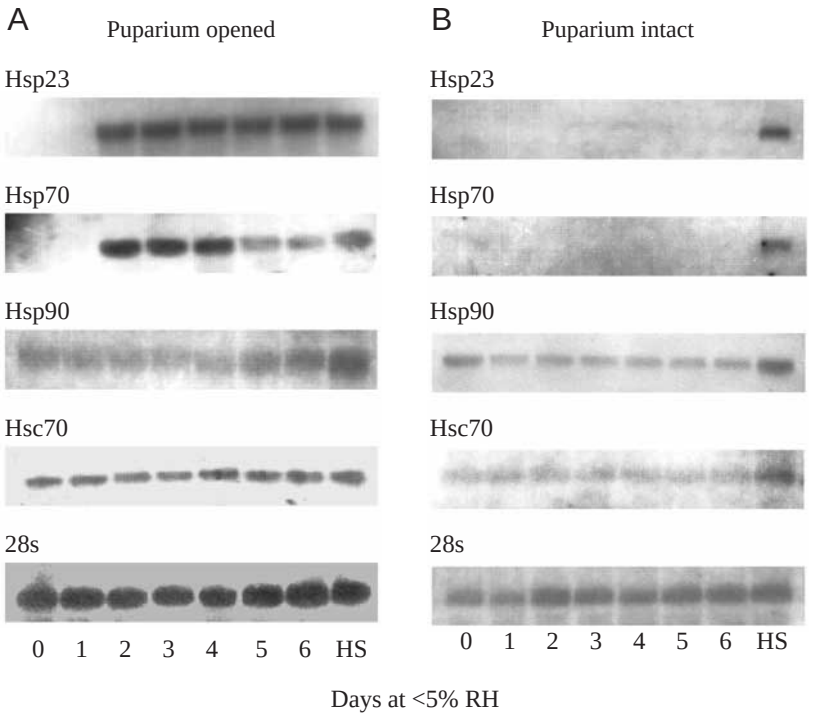

Fig. 2. Expression of *hsp23*, *hsp70*, *hsp90* and *hsc70* transcripts in nondiapausing pupae in response to desiccation. Pupae were encased in either (A) opened or (B) intact puparia. Lanes represent RNA samples from pupae desiccated at <5% RH/25°C for 0–6 days or exposed to a heat shock (HS) of 1 h at 40°C. Each sample was run in triplicate. 28s ribosomal RNA was used as a control to confirm equal sample loading.

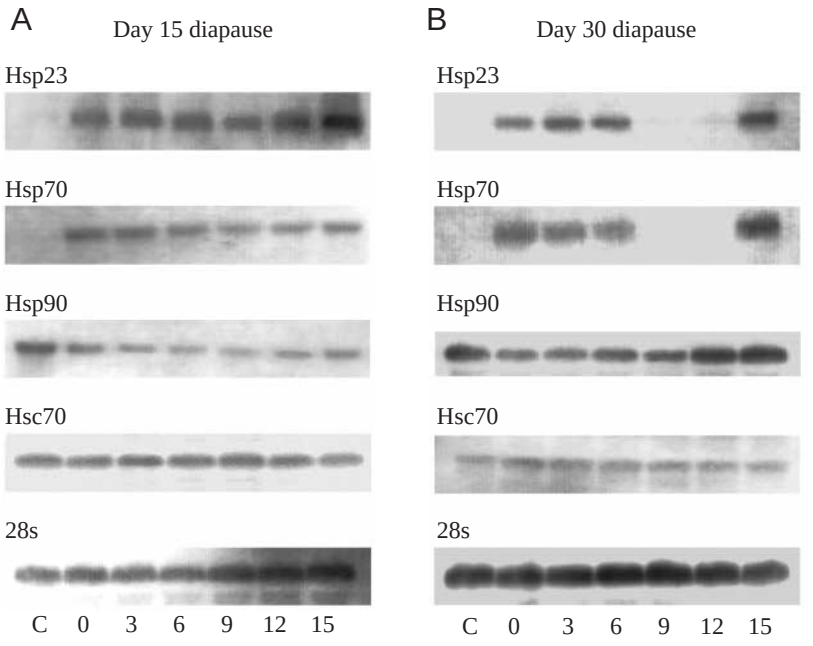

Fig. 3. Expression of *hsp23*, *hsp70*, *hsp90* and *hsc70* transcripts in (A) day 15 and (B) day 30 diapausing pupae. The puparium was opened in all cases. C represents RNA from a nondesiccated, nondiapausing control, and other lanes indicate the number of days that diapausing pupae were exposed to desiccation (<5% RH/25°C). Each sample was run in triplicate. 28s ribosomal RNA was used as a control to confirm equal sample loading.

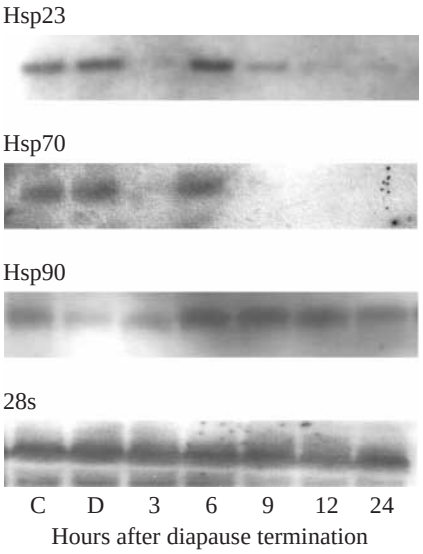

Fig. 4. Expression of *hsp23*, *hsp70* and *hsp90* transcripts during diapause termination in day 15 diapause pupae previously desiccated at <5% RH/25°C for 9 days. C represents RNA from a nondesiccated diapause control sample. D represents a desiccated diapause sample (9 days at <5% RH). Other lanes represent hours following the termination of diapause elicited by an application of hexane. Each treatment was replicated at least twice. 28s ribosomal RNA was used as a control to confirm equal sample loading.

contrast, hsp90 and hsc70 were both upregulated by rehydration in both nondiapausing (Fig. 6A) and diapausing (Fig. 6B) pupae.

Discussion

Several earlier studies report the upregulation of Hsps in response to desiccation stress in insects (Tammariello et al., 1999; Bayley et al., 2001) but, to the best of our knowledge, the present study provides the first evidence showing a distinct Hsp response to rehydration. Like the study on *S. crassipalpis* by Tammariello et al. (1999), we observed the upregulation of Hsp23 and Hsp70 in nondiapausing pupae following desiccation stress. We have now also evaluated the response of Hsp90 and Hsc70 (the Hsp70 cognate) to desiccation stress and found that desiccation does not elicit the upregulation of either. However, both Hsp90 and Hsc70 are upregulated when the fly pupae are rehydrated following a period of dehydration.

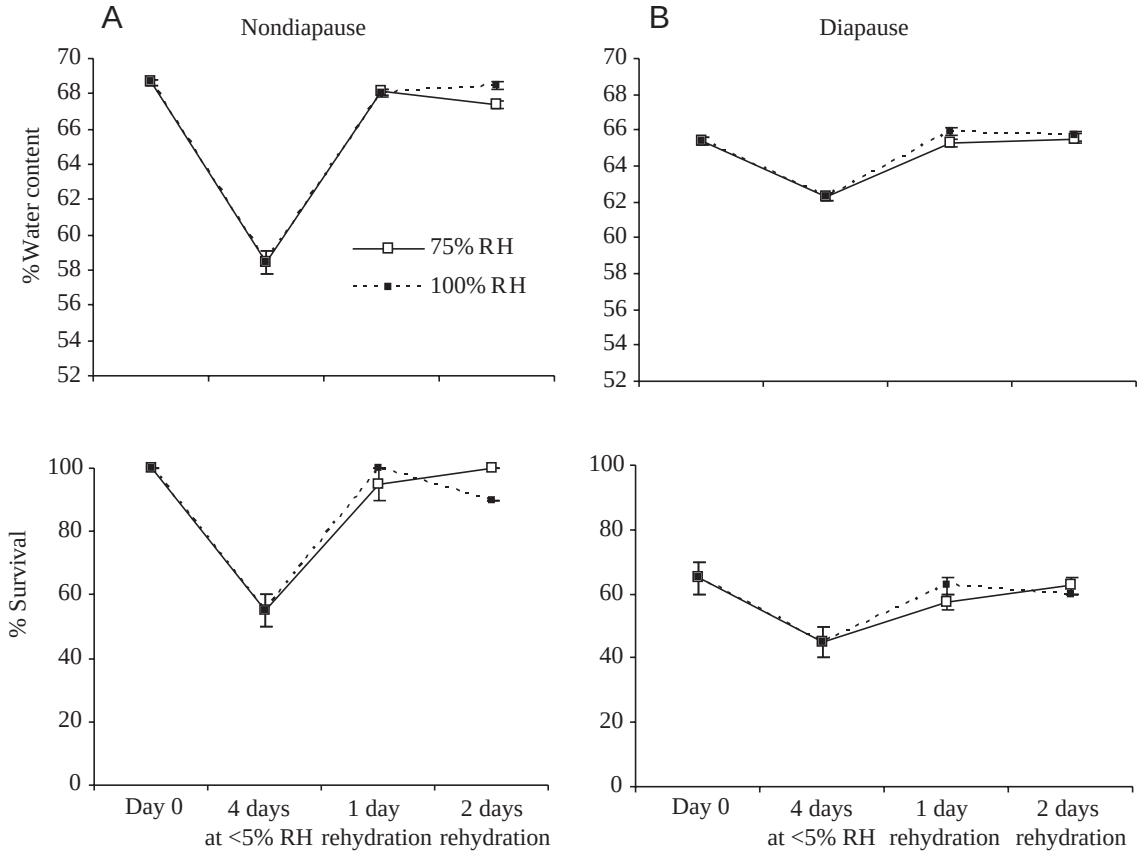

Fig. 5. Water content and survival in (A) nondiapausing and (B) diapausing pupae desiccated at <5% RH for 4 days and then rehydrated at either constant 75% or 100% RH for 1 or 2 days. Water content is given as a percentage of the initial fresh mass (N=20 for each time point), and percent survival is based on N=20 for each time point. Means ± S.E.M. are presented. Day 0 represents the start of each desiccation treatment for either day 4 nondiapausing pupae or day 15 diapausing pupae.

Thus, one set of Hsps is upregulated in response to desiccation and another to rehydration (recovery from desiccation). These results imply distinct functions for the different stress proteins, as has been noted in several previous reports.

While many of the Hsps are simultaneously upregulated in response to heat (Lindquist and Craig, 1988), other forms of stress may selectively induce different Hsps. For example, in *S. crassipalpis*, constitutively expressed Hsp90 is further upregulated in response to heat shock (40°C) but does not respond directly to cold treatment (–10°C; Rinehart and Denlinger, 2000). By comparison, Hsc70, which is also constitutively expressed, is not immediately upregulated by either cold or heat treatment in *S. crassipalpis* (Rinehart et al., 2000). However, both of these constitutive Hsps are upregulated during the recovery from cold shock, demonstrating considerable similarity to their response to rehydration/desiccation recovery identified in the present study. This result indicates another common link between adaptations to cold and desiccation stress.

Desiccation and rehydration create contrasting cellular environments, which could help explain their different Hsp responses. To compensate for the deficit in hydrogen bonding with water caused by cellular dehydration, hydrogen bonding with other molecules can occur, which may lead to protein aggregation and/or denaturation (Pestrelski et al., 1993). Water loss from membrane phospholipids also leads to phase transitions from the biologically active fluid phase to the gel phase (Crowe et al., 1992), which in turn influences transmembrane ion and protein activity. A principal role for both small Hsps and Hsp70 is to bind to denatured proteins and inhibit aggregation (Fink, 1999). Small Hsps are also thought to function in stabilizing the liquid crystalline state of membranes (Tsvetkova et al., 2002), and membrane lipid interactions with Hsp70 have been identified (Arispe et al., 2002). By contrast, rehydration will increase cellular turgor pressure and re-establish membrane fluidity, as well as re-initiate productive protein folding pathways, in which both Hsc70 (Leung and Hightower, 1997) and Hsp90 (Young et al., 2001) play a fundamental role. Despite rehydrated samples returning to their pre-desiccated water content within a 24 h period (Fig. 5), the reversal of membrane disruption and the re-establishment of protein folding often takes considerably longer (Bernacchia et al., 1996). This could explain the prolonged expression of Hsp90 and Hsc70 during the rehydration phase.

Clearly, Hsps are not the only players likely to be involved in desiccation/rehydration responses of insects. A desiccation-specific protein has been reported from the mealworm *Tenebrio molitor* (Kroeker and Walker, 1991a,b). Although its identity remains unknown, it does not appear to be an Hsp. Plants also possess different classes of desiccation and rehydration responsive proteins, termed dehydrins and rehydrins, respectively, along with their seed counterparts, the late-embryogenesis-abundant (LEA) proteins (Bohnert, 2000). While these proteins have functional similarity to certain Hsps (Mtwisha et al., 1998), they are distinct and give an indication of diverse molecular responses to desiccation/rehydration stress in terrestrial organisms.

Polyols, sugars and other such cryoprotectants are also likely to contribute to the insect's response to desiccation (Bayley and Holmstrup 1999). This appears to be true for *S. crassipalpis*: glycerol synthesis increases in response to desiccation in nondiapausing pupae (S.A.L.H., unpublished observations). Interactions between glycerol/trehalose and Hsps have been well documented in systems as diverse as human cell cultures (Brown et al., 1996) and the brine shrimp *Artemia franciscana* (Viner and Clegg, 2001). Interactions between membrane lipids and Hsps are also well documented (Tsvetkova et al., 2002; Arispe et al., 2002), thus underscoring the likelihood that Hsps are unlikely to be responding to desiccation/rehydration stress in isolation from other cell processes.

An interaction between Hsps and other physiological stress mechanisms could help explain the difference in Hsp transcription thresholds between desiccated, intact, nondiapausing pupae and nondiapausing

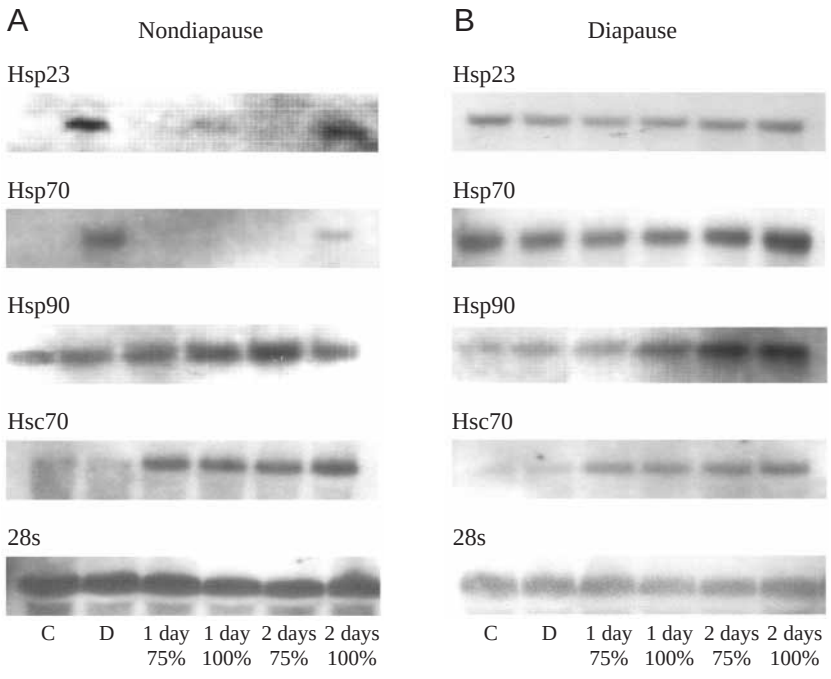

Fig. 6. Expression of *hsp23*, *hsp70*, *hsp90* and *hsc70* transcripts in response to the rehydration of desiccated (A) nondiapausing and (B) day 15 diapausing pupae. C represents RNA from a nondesiccated control. D represents pupae desiccated for 4 days at <5% RH. Remaining lanes represent pupae rehydrated for 1 or 2 days at 75% or 100% RH. Each treatment was run in triplicate. 28s ribosomal RNA was used as a control to confirm equal sample loading.

samples desiccated with the operculum of their puparium removed. After 6 days at <5% RH/25°C, intact, nondiapausing pupae lose more than 10% of their initial water content (Fig. 1A). This is beyond the water loss threshold that initiates *hsp* transcription in nondiapause samples with an open puparium, yet neither Hsp23 nor Hsp70 is upregulated in the intact group (Fig. 2B). Thus, the threshold of Hsp expression increases in response to a reduced rate of desiccation. The rate of water loss is known to influence cryoprotectant synthesis (Bayley and Holmstrup, 1999) and cell membrane adaptation (Holmstrup et al., 2002) in some terrestrial arthropods, but how this may influence Hsp expression has not been investigated. Biochemical analysis of cryoprotectant synthesis and membrane lipid composition during dehydration in insects, and its influence on Hsp expression, therefore seems an appropriate avenue for future research.

Our experiments with *S. crassipalpis* also revealed major differences in responses to desiccation stress between diapausing and nondiapausing pupae and underscore the role of the puparium in offering protection against dehydration. Under natural conditions, these flies enter diapause in the early autumn and the adult flies do not emerge from the puparium until the following spring (Denlinger, 1972). During these many months, the fly does not have access to free water, and the maintenance of water balance emerges as a critical issue (Yoder and Denlinger, 1991). The addition of an extra layer of hydrocarbons on the surface of the puparium provides an important barrier to water loss (Yoder et al., 1992), and from the present study we can see the impact of breaching that barrier by removing the operculum and thus exposing the pupal body to the atmosphere. The rate of water loss was dramatically increased in these opened puparia, yet the impact of opening the puparium differs between diapausing and nondiapausing pupae. Water loss rates remained far lower in diapausing pupae with an opened puparium than in their nondiapausing counterparts, presumably due to the suppressed metabolism (Denlinger et al., 1972) and elevated glycerol content (Lee et al., 1987) inherent in the diapause program.

Differences in the Hsp response to desiccation and rehydration are also evident between these two types of pupae. While the abundance of *hsp23* and *hsp70* transcripts increased in response to the desiccation of nondiapausing pupae (operculum removed), both transcripts were unresponsive to desiccation in diapausing pupae. This is probably because both hsp23 and hsp70 are already highly expressed in diapausing pupae by virtue of their being in diapause (Denlinger et al., 2001). The developmental upregulation of these Hsps during diapause possibly represents a prophylactic response for diapausing pupae against desiccation, temperature extremes and other stresses to which the overwintering pupa may be exposed (Denlinger et al., 2001). The unresponsiveness of hsp23 and hsp70 during diapause is further highlighted by their sustained expression during the rehydration of desiccated diapausing pupae (Fig. 6B). This contrasts with rehydrated nondiapausing pupae, in which both *hsp23* and *hsp70* transcripts decline (Fig. 6A).

Desiccation also elicited another, somewhat unexpected, response on development time. Three or more days at <5% RH extended the interval between pupariation and adult eclosion by several days in nondiapausing flies (Fig. 1A). This duration of stress was sufficient to elicit expression of hsp23 and hsp70, and we thus anticipate that this upregulation of Hsps could contribute to the delay in adult eclosion, based on previous work indicating that the expression of Hsps is not compatible with the progression of development (Feder and Hofmann, 1999). Indeed, one of the first events associated with pupal diapause termination and the initiation of adult development in flesh flies is downregulation of the Hsps (Yocum et al., 1998; Rinehart et al., 2000). A similar response was noted in the present study when older diapausing pupae started to break diapause during desiccation. In such pupae, Hsp70 and Hsp23 synthesis was interrupted during diapause termination despite samples being under desiccation stress. A couple of days later, after development had been initiated, the flies again expressed the *hsp* transcripts if they remained in a desiccating environment (Fig. 3). This association was tested further using hexane as a tool to terminate diapause under desiccating conditions. The fact that desiccated diapausing pupae that were stimulated to break diapause with hexane ceased to express Hsps (Fig. 4), even though they continued to be exposed to desiccation stress, is consistent with the idea that synthesis of stress-induced Hsps must stop before development can ensue.

In summary, this paper demonstrates that the expression of some, but not all, Hsps is elicited by desiccation, and a different set of Hsps responds to rehydration. Pupae in diapause already express the Hsps elicited by desiccation and no further upregulation is noted. Several lines of evidence also suggest a causal relationship between the desiccation-stimulated upregulation of Hsps and the delay in development observed in such pupae.

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