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Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much?

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1. Introduction

Inducible heat shock proteins (Hsps) and other molecular chaperones vary in concentration within cells. In insects, for example, Hsp70 is virtually absent from unstressed cells (Velazquez et al., 1983) but within minutes can account for the bulk of protein synthesis (Loomis and Wheeler, 1982; Palter et al., 1986). Differences in the concentrations of Hsps that cells produce have several consequences (reviewed in Feder and Krebs, 1997a); these mainly concern interactions with other proteins. When proteins unfold at high temperatures, they expose internal residues that are ordinarily isolated from one another in the native state. These residues can interact to form aggregates, which harm or kill the cell. At low to moderate concentrations, Hsps, behaving as molecular chaperones, can recognize and bind to these unfolded or non-native proteins (Lindquist and Craig, 1988; Parsell et al., 1993; Morimoto et al., 1994) and minimize these aggregations (Parsell and Lindquist, 1993). At supra-physiological concentrations, by contrast, Hsps can bind proteins inappropriately, interfere with their cellular localization (Dorner et al., 1992), and harm organismal growth and development (Krebs and Feder, 1997a). Therefore, precise and effective regulation of Hsp concentrations is especially critical. To examine this point, we experimentally manipulated concentrations of a single Hsp, the inducible 70-kD Hsp family member of *Drosophila melanogaster*, and here report the consequences of this manipulation for susceptibility to thermal injury and organismal thermotolerance.

In the past, relating Hsp concentration to thermal tolerance has proceeded along several lines. Descriptive research suggested a quantitative and chronological correspondence between Hsp levels and inducible thermo-

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tolerance (Stephanou et al., 1983; Velazquez and Lindquist, 1984; McColl et al., 1996; Krebs and Feder, 1997b; DiIorio et al., 1996), and between the decline of Hsps, mRNA for heat-shock proteins, and recovery of normal protein synthesis in *Drosophila* cells (DiDomenico et al., 1982a, b). Genetic engineering, which enabled investigators to vary Hsp expression against a constant genetic background, established an unambiguous qualitative relationship between Hsp70 level and inducible thermotolerance in insect cells in culture (Li et al., 1991; Solomon et al., 1991; Li and Duncan, 1995) and in whole insects, after insertion of additional *hsp70* gene copies into *Drosophila* lines (Welte et al., 1993). Continued research on the extra-copy and their transcript-excised control lines indicated that higher Hsp70 expression can lengthen survival to heat stress at specifi points in development (Feder et al., 1996). Also, removal of Hsp70 by introduction of anti-Hsp70 antibodies disrupts transcription (Moreau et al., 1994) and the development of tolerance to heat (Riabowol et al., 1988; Solomon et al., 1991; Lee et al., 1993).

We may now expand on earlier work, which compared effects of Hsp70 over-expression primarily under those conditions that maximized Hsp70 concentrations within flie (Feder et al., 1996; Krebs and Feder, 1997a), and manipulate Hsp70 expression quantitatively (1) to elucidate the relevance of Hsp expression to natural populations, which encounter diverse temperatures and rates of thermal increase, (2) to characterize the physiology of Hsp expression in different tissues (e.g. Krebs and Feder, 1997c) and (3) to provide insight to the evolution of regulatory control (e.g., Lindquist, 1993) of Hsp concentrations via putative trade-offs between the benefit and costs of high expression. First, *Drosophila* larvae and pupae can encounter quite severe thermal stress in nature (Feder et al., 1997) but clearly not all larvae and pupae encounter such stress. Hsp expression, moreover, is not instantaneous, and some time must elapse between a mild Hsp-inducing stress and severe stress for Hsp expression to promote tolerance. Accordingly, questions arise as to how mild and how brief an Hsp-inducing stress can be and still benefi an insect, and whether natural levels of Hsps are sufficien for inducible thermotolerance. Second, despite the compendious literature on Hsps (e.g., Lindquist, 1986; Gething and Sambrook, 1992; Morimoto et al., 1994), exactly how the proteins cause entire organs and organisms to become thermotolerant is not well understood. Underlying mechanisms can become obvious by comparing organs and individuals that differ in the kinetics of Hsp expression. The larval midgut and gastric caeca, for example, are especially vulnerable to heat and exhibit slow kinetics of Hsp70 expression and recovery (Krebs and Feder, 1997c). Finally, as intimated above, Hsp70 can be neutral or beneficia at intermediate concentrations but harmful at high concentrations or when it is expressed constitutively (Feder et al., 1992; Krebs and Feder, 1997a). Contrasting benefit and costs may limit expression and influenc the evolution of regulatory controls (Krebs and Feder, 1997b). If so, manipulating Hsp70 concentrations may elucidate costs, benefits and any resultant trade-off.

2. Materials and methods

2.1. Strain origin

We characterized effects of varying conditioning treatments (pretreatment) on larval thermotolerance in a pair of strains engineered to vary in *hsp70* copy number and, consequently, expression of Hsp70. Welte et al. (1993) described the construction of the transgenic strains via unequal homologous recombination. The extra-copy strain contains a pair of transgene constructs, each a composite of three *hsp70* genes, an eye color marker, w^{hs}, and flankin yeast recombination targets and P-elements. The excision strain shares the same chromosomal site of transgene integration and flankin elements, but lacks the *hsp70* transgenes and eye color marker. The procedure that duplicates the initial insert in one chromatid simultaneously deletes the insert in another, thereby producing two strains varying in copy number but possessing similar genetic backgrounds (details in Feder et al., 1996; Krebs and Feder, 1997a). One member of the pair, the extra-copy strain, has 12 *hsp70* transgenes in addition to its 10 natural copies (Ish-Horowicz et al., 1979a, b). The other member, the excision strain, has only the 10 natural copies but shares the transgene integration site with the extra-copy strain, and thus is a control for insertional mutagenesis. For the strains used here, the site of transgene integration mapped to chromosome II.

2.2. Pretreatment and heat shock

Before analyses of Hsp70, larval fitnes and tissue damage, we exposed larvae to one of a graded series of Hsp70-inducing pretreatments, heat shock and/or recovery at 25°C. The heat shock was 60 min at 39.2°C for 1st-instar larvae or 60 min at 38.5°C for 3rd-instar larvae, which are less thermotolerant than 1st-instars (Krebs and Feder, 1997a). Pretreatments for Hsp70 and fitnes analyses were for 10, 30 and 60 min at 36°C and then 60 min at 25°C, or at 32, 33, 34, 35, and 36°C for 60 min and then 60 min at 25°C. Samples were preserved in liquid N_2 or at -80°C either after the pretreatment or after heat shock. Pretreatment of 3rd-instar larvae was for 12 min or 60 min at 36°C and then 60 min at 25°C, with individuals either scored immediately or allowed 21 h recovery.

2.3. Hsp70 determination

Hsp70 concentration was determined by enzyme linked immunosorbent assay (ELISA), which has been detailed elsewhere (Welte et al., 1993; Feder et al., 1996; Krebs and Feder, 1997b). Results were expressed as a percent of a standard, expression in S2 *Drosophila* cells treated at 36.5°C for 60 min and 60 min at 25°C. Treatment and handling of larvae used in Hsp70 quantificatio varied according to developmental stage. First-instar larvae were collected from the medium surface and 3rd-instar larvae were separated from the medium according to Ashburner (1989). Hsp70 determination required 40 larvae in the 1st-instar stage, collected 4–12 hours after hatching, to obtain sufficien protein levels, or three 3rd-instar larvae, collected 6 days after laying. First-instar larvae were placed in microfuge tubes with 10 μl phosphate-buffered saline (PBS), submerged in thermostatted water baths and then frozen in liquid N_2. Hsp70 is not present in the absence of heat shock (Velazquez et al., 1984; Feder et al., 1996), nor do larvae produce Hsp70 > 2% of the standard at 39.2°C or higher unless they receive pretreatment (Krebs and Feder, 1997c). For heat treatment of 3rd-instar larvae, we transferred larvae to 5 cm petri dishes that contained medium, sealed them with Parafil ©, and immersed them in a water bath at the required temperature.

Because gut tissue is especially sensitive to heat shock and damage is correlated with chronic elevation of Hsp70 expression (Krebs and Feder, 1997c), we also determined Hsp70 concentration in pooled midguts (including caeca). To collect tissue, we peeled away epidermis and muscle to expose the body cavity, and removed the midgut between the proventriculus and the Malpighian tubules. For each sample, we dissected 5 midguts in ice-cold PBS, which required 6–9 minutes before freezing in liquid N_2.

2.4. Larval fitness

To determine larva-to-adult survival and developmental time, 40 first-insta larvae per replicate were transferred to glass vials containing 8 ml of a yeast-cornmeal-molasses-agar medium. When not undergoing treatment, all larvae developed in these food vials within a large humidifie container at 25°C. Each day we collected the adults that emerged from vials. Thermotolerance was estimated as the survival proportion after a 39.2°C heat shock, standardized to the proportion emerging at 25°C for each line. These standardized survival data underwent arcsine square-root transformation before statistical analysis. Mean developmental time per vial was the average of that for males and females, each determined as $\log^{-1}$ of the log day-of-emergence per individual.

2.5. Trypan Blue staining

Assays of tissue damage utilized Trypan Blue, which is excluded from healthy cells but readily enters dead or damaged cells (Krebs and Feder, 1997c), marking alteration in membrane structure (Bowler and Manning, 1994, p. 197). Heat-treated larvae were placed in PBS at room temperature, the cuticle and muscle tissue peeled from the body cavity, the internal tissues immersed in 0.2 mg ml^{-1} Trypan Blue in PBS, and then rotated for 30 min at room temperature (23°C) to bring all tissues in contact with dye. After staining, larvae were rinsed three times in PBS, washed for 30 min in PBS, and each larva was immediately scored for Trypan Blue content in tissues and cells: no color, 0; any blue, 1; darkly stained nuclei, 2; large patches of darkly stained cells, 3; or complete staining of most cells in the tissue, 4. As these data are sequential categories, differences due to strain (extra-copy vs. excision), treatment effects (pretreated vs. no pretreat, short pretreatment vs. long pretreatment) and recovery time (within 2 h after heat shock vs. 21 h after heat shock) were tested by Mann-Whitney U-tests.

3. Results

3.1. 1st-instar larvae — Hsp70

Pretreatment duration and intensity affected Hsp70 concentration (Fig. 1A, Fig. 2A). After pretreatment at 36°C, Hsp70 concentrations increased during the 39.2°C heat shock, a change that did not occur without pretreatment. At all pretreatment durations and temperatures, extra-copy larvae produced more Hsp70 than did excision larvae ($P < 0.001$).

3.2. 1st-instar larvae — mortality and developmental time

Lengthening pretreatment duration at 36°C generally increased larval thermotolerance (Fig. 1B, $F_{2,90} = 52$, $P < 0.001$). Extra-copy larvae were more thermotolerant than excision larvae in the absence of pretreatment and with a short (10 min) pretreatment. Excision larvae, however, were more thermotolerant where larvae received 30 min pretreatments (strain × pretreatment duration interaction, $F_{2,90} = 3.3$, $P < 0.05$). Thus, Hsp70 level before heat shock and tolerance to the heat shock were correlated at low to moderate Hsp70 concentrations, but inversely related at high Hsp70 concentrations.

We also manipulated Hsp70 level by varying pretreatment temperature at a constant duration (Fig. 2A, $F_{4,30} = 8.0$, $P < 0.001$). Increasing pretreatment temperature increased thermotolerance (Fig. 2B, $F_{4,30} = 8.0$, $P <$

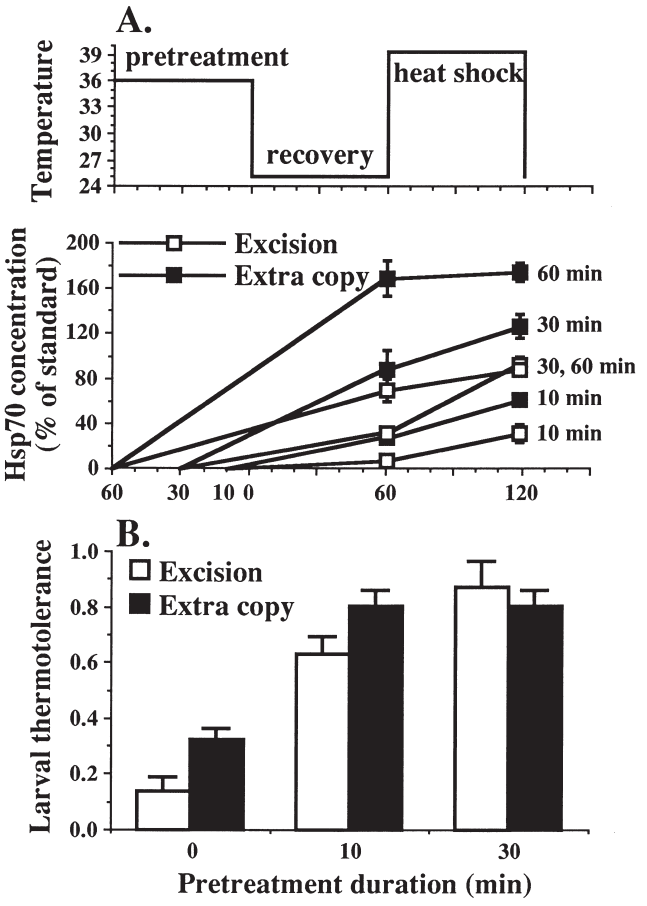

Fig. 1. Effect of pretreatment duration on Hsp70 accumulation and thermotolerance of 1st-instar larvae. A shows temperatures during pretreatment and heat shock and Hsp70 levels following each of the three pretreatment durations at 36°C. Each point represents the mean of 4 samples each of 40 larvae. B shows larva-to-adult survival after larvae underwent the temperature regimes depicted, with survival standardized to control levels (that at continuous 25°C). Sample sizes were 640 larvae per treatment group, 40 in each of 16 vials. We measured Hsp70 levels by ELISA in whole-body lysates using a monoclonal antibody specifi for Hsp70. Results are expressed relative to the signal obtained with a standard derived from heat-shocked *Drosophila* tissue culture cells (see Materials and Methods).

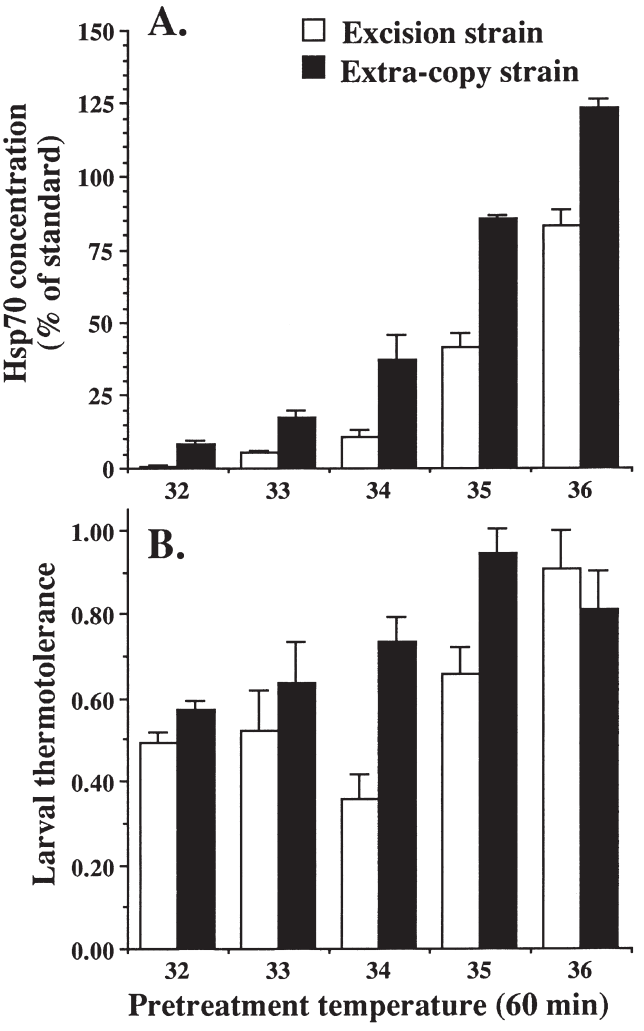

Fig. 2. Effect of pretreatment temperature on Hsp70 accumulation and thermotolerance of 1st-instar larvae. A shows Hsp70 levels following a 60 min exposure to different temperatures and 60 min at 25°C (sample sizes of 4 replicates each of 40 larvae). B shows larva-to-adult survival after a 60 min heat shock at 39.2°C for larvae pretreated 60 min at the indicated temperature followed by 60 min at 25°C. Survival was standardized to control levels (that at continuous 25°C), and sample sizes were 160 larvae per treatment group, 40 in each of 4 vials).

0.001), as did changes in pretreatment duration. Extra-copy larvae were more thermotolerant than excision larvae after pretreatment at 35°C or less. After 1 h at 36°C, the concentration of Hsp70 exceeded that possible in the excision line, which has normal gene-copy numbers (Fig. 2A), and thermotolerance of these excision larvae exceeded that of extra-copy larvae (strain × temperature, $F_{4,30} = 2.9$, $P < 0.05$).

Heat shock delayed development of larvae relative to 25°C controls if they received no pretreatment (Fig. 3A) or pretreatment at 32°C (Fig. 3B). Pretreatments of 36°C for > 10 min or 60 min at 33°C or higher prevented the delay in development. Therefore, developmental time varied little due to pretreatment duration ($F_{3,71} = 2.5$, $P < 0.1$) and temperature ($F_{5,41} = 2.0$, $P < 0.1$).

3.3. 3rd-instar larvae — Hsp70

As in 1st-instar larvae, increasing pretreatment duration at 36°C in 3rd-instar larvae increased Hsp70 concentrations both before heat shock (Fig. 4A, $P < 0.001$) and after a subsequent 38.5°C heat shock ($P < 0.001$). The increase in extra-copy larvae exceeded that in excision larvae ($P < 0.001$), and the relative response during heat shock by extra-copy and excision larvae differed depending on pretreatment duration. After a 12 min pretreatment, extra copy larvae produced more Hsp70 during heat shock than after a 60 min pretreatment, while excision larvae produced more Hsp70 after the 60 min than after the 12 min pretreatment (strain × pretreatment duration × heat shock, $P < 0.05$).

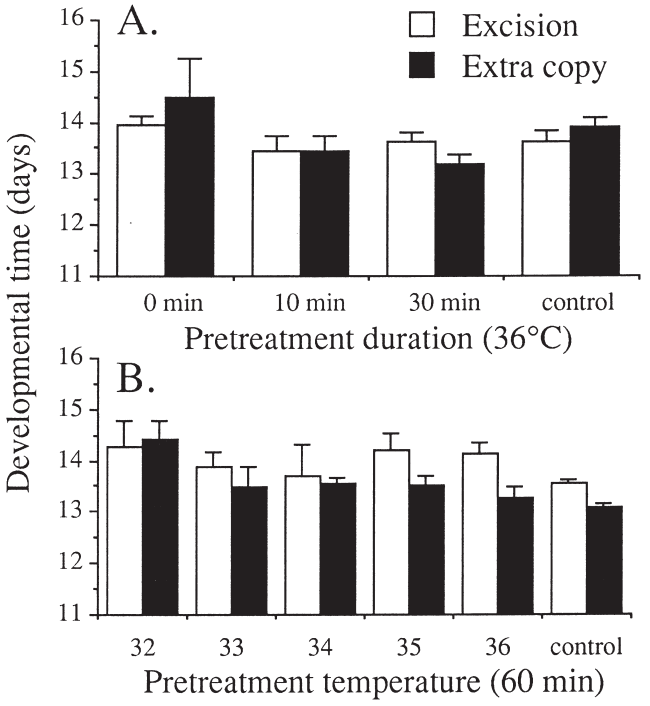

Fig. 3. Effect of pretreatment duration and temperature on time from 1st-instar larva to adult. Except for controls reared at 25°C, larvae were pretreated and heat shocked for 60 min at 39.2°C. A shows larvae used in Fig. 1, which were pretreated at 36°C for varying lengths of time followed by 60 min at 25°C before heat shock. B shows larvae used in Fig. 2, which were pretreated by 60 min exposure at different temperatures also followed by 60 min at 25°C before heat shock.

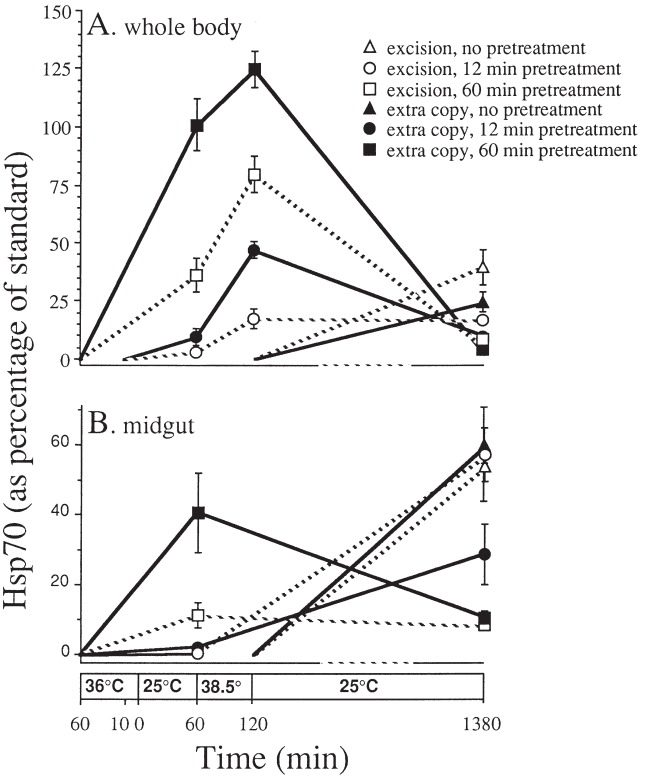

Fig. 4. Hsp70 accumulation in whole 3rd-instar larvae (A) and specificall midgut (B) from the extra-copy (fille symbols) and excision (open symbols) strains after either a 36°C pretreatment and/or a 38.5°C heat shock. Hsp70 level in the absence of any heat treatment or 21 h after pretreatment only is negligible (results not shown). Each point represents the mean of 4–6 samples each including either 2–3 whole larvae or 5 gut dissections.

Higher Hsp70 levels before heat shock correlated inversely with levels of Hsp70 a day after this treatment (Fig. 4A). Hsp70 levels 21 h after heat shock were highest in larvae not pretreated before heat shock, intermediate in larvae pretreated 12 min before heat shock, and lowest in larvae pretreated 60 min before the heat shock. This relationship between Hsp70 concentration before and after heat shock held for both strains. Although Hsp70 concentrations typically are higher in extra-copy larvae immediately after pretreatment and heat shock, levels 21 h after heat shock in excision larvae exceeded those in the extra-copy larvae.

Concentrations of Hsp70 in midgut and whole-body lysates differed, but strain differences and temporal variation in expression followed similar patterns in both analyses (Fig. 4). Midguts contained lower Hsp70 concentrations after pretreatment than did whole body lysates, and levels after 21 h in this tissue exceeded those in whole larvae. Extra-copy larvae expressed more Hsp70 in the gut immediately after pretreatment than did excision larvae ($F_{1,17} = 4.0$, $P < 0.05$), but strain differences were small a day later both in the absence of pretreatment and after the 60 min pretreatment. Because midgut produces Hsp70 slowly, concentrations in midgut after the 12 min pretreatment were very low, but were higher in extra-copy larvae than in excision larvae. Excision larvae subsequently had the higher concentrations 21 h after heat shock (strain × pretreatment duration, $F_{2,20} = 3.74$, $P < 0.05$). Thus in a single tissue, pre-heat shock concentrations of Hsp70 and concentrations 21 h after heat shock are inversely related, as in whole larvae.

3.4. 3rd instar larvae — Trypan Blue

We directly assessed the amount of Trypan Blue incorporated by cells after heat shock; healthy cells exclude this dye. Control larvae and those pretreated for 60 min at 36°C stained little (data not shown). Heat shock, by contrast, increased staining in all tissues above that of controls, both < 2 h after heat shock (hereafter "acutely") and after a 21 h recovery (Table 1). Gut tissues, particularly the caeca and midgut, stained far more than did other tissues (Fig. 5; and see Krebs and Feder, 1997c, Figure 7).

Pretreatment failed to ameliorate acute heat-induced damage. Heat-shocked larvae stained similarly regardless of whether they received a 60 min pretreatment at 36°C or no pretreatment (Table 1), and neither did the excision or extra-copy strains vary in Trypan Blue staining < 2 h after heat shock (Fig. 5).

Table 1
Quantificatio of Trypan Blue staining after heat shock in various tissues of 3rd-instar extra-copy and excision larvae

Tissue type	Strain	Recovery Time	Trt	Trypan Blue scoring N	0	1	2	3	4
Brain	Excision	2 h	NP	29	0.90	0.07	0.03	0.00	0.00
			P60	29	0.97	0.03	0.00	0.00	0.00
		21 h	NP	33	1.00	0.00	0.00	0.00	0.00
			P12	44	1.00	0.00	0.00	0.00	0.00
			P60	26	0.96	0.04	0.00	0.00	0.00
	Extra copy	2 h	NP	28	0.96	0.04	0.00	0.00	0.00
			P60	33	0.94	0.06	0.00	0.00	0.00
		21 h	NP	43	0.91	0.07	0.02	0.00	0.00
			P12	58	0.98	0.02	0.00	0.00	0.00
			P60	25	0.96	0.00	0.04	0.00	0.00
Salivary glands	Excision	2 h	NP	24	0.71	0.04	0.17	0.04	0.04
			P60	21	0.76	0.05	0.14	0.05	0.00
		21 h	NP	28	0.82	0.00	0.18	0.00	0.00
			P12	38	0.79	0.05	0.16	0.00	0.00
			P60	23	0.65	0.13	0.17	0.04	0.00
	Extra copy	2 h	NP	19	0.74	0.11	0.11	0.05	0.00
			P60	33	0.67	0.21	0.12	0.00	0.00
		21 h	NP	37	0.65	0.11	0.22	0.03	0.00
			P12	51	0.76	0.02	0.22	0.00	0.00
			P60	31	0.71	0.14	0.10	0.05	0.00
Proventricules	Excision	2 h	NP	28	0.82	0.07	0.11	0.00	0.00
			P60	30	0.93	0.03	0.03	0.00	0.00
		21 h	NP	32	0.72	0.03	0.22	0.03	0.00
			P12	44	0.95	0.00	0.05	0.00	0.00
			P60	27	0.96	0.00	0.04	0.00	0.00
	Extra copy	2 h	NP	26	0.84	0.04	0.12	0.00	0.00
			P60	35	0.89	0.03	0.09	0.00	0.00
		21 h	NP	42	0.74	0.07	0.19	0.00	0.00
			P12	58	0.97	0.00	0.03	0.00	0.00
			P60	23	0.87	0.09	0.04	0.00	0.00
Hindgut	Excision	2 h	NP	30	0.57	0.03	0.40	0.00	0.00
			P60	30	0.73	0.20	0.07	0.00	0.00
		21 h	NP	33	1.00	0.00	0.00	0.00	0.00
			P12	44	0.93	0.02	0.05	0.00	0.00
			P60	27	0.74	0.11	0.15	0.00	0.00
	Extra copy	2 h	NP	25	0.52	0.04	0.44	0.00	0.00
			P60	35	0.74	0.11	0.14	0.00	0.00
		21 h	NP	41	0.80	0.07	0.12	0.00	0.00
			P12	58	0.84	0.07	0.09	0.00	0.00
			P60	25	0.80	0.08	0.12	0.00	0.00
Malpigian tubules	Excision	2 h	NP	30	0.70	0.13	0.17	0.00	0.00
			P60	30	0.73	0.17	0.10	0.00	0.00
		21 h	NP	33	0.52	0.24	0.24	0.00	0.00
			P12	44	0.95	0.02	0.02	0.00	0.00
			P60	27	0.74	0.07	0.11	0.07	0.00
	Extra copy	2 h	NP	28	0.89	0.11	0.00	0.00	0.00
			P60	35	0.69	0.20	0.11	0.00	0.00
		21 h	NP	43	0.63	0.23	0.14	0.00	0.00
			P12	58	0.84	0.07	0.09	0.00	0.00
			P60	25	0.76	0.20	0.04	0.00	0.00
Fat bodies	Excision	2 h	NP	30	0.40	0.27	0.30	0.03	0.00
			P60	30	0.60	0.20	0.13	0.07	0.00
		21 h	NP	33	0.70	0.09	0.21	0.00	0.00
			P12	44	0.80	0.09	0.07	0.05	0.00
			P60	27	0.81	0.11	0.07	0.00	0.00
	Extra copy	2 h	NP	28	0.61	0.18	0.33	0.00	0.00
			P60	35	0.74	0.23	0.03	0.00	0.00
		21 h	NP	43	0.70	0.12	0.14	0.05	0.00
			P12	58	0.78	0.07	0.12	0.03	0.00
			P60	25	0.76	0.12	0.12	0.00	0.00

NP, No pretreatment before 38.5°C heat shock; P12, 12 min at 36°C and 60 min at 25°C before heat shock; P60, 60 min at 36°C and 60 min at 25°C before heat shock. Scoring of larval tissues occurred either within 2 h of heat shock or after a recovery of 21 h at 25°C: (0) no color; (1) any blue; (2) darkly stained nuclei; (3) large patches of darkly stained cells; (4) complete staining of most cells in the tissue. Reported are the number of individuals measured for each tissue and the frequency in each staining class.

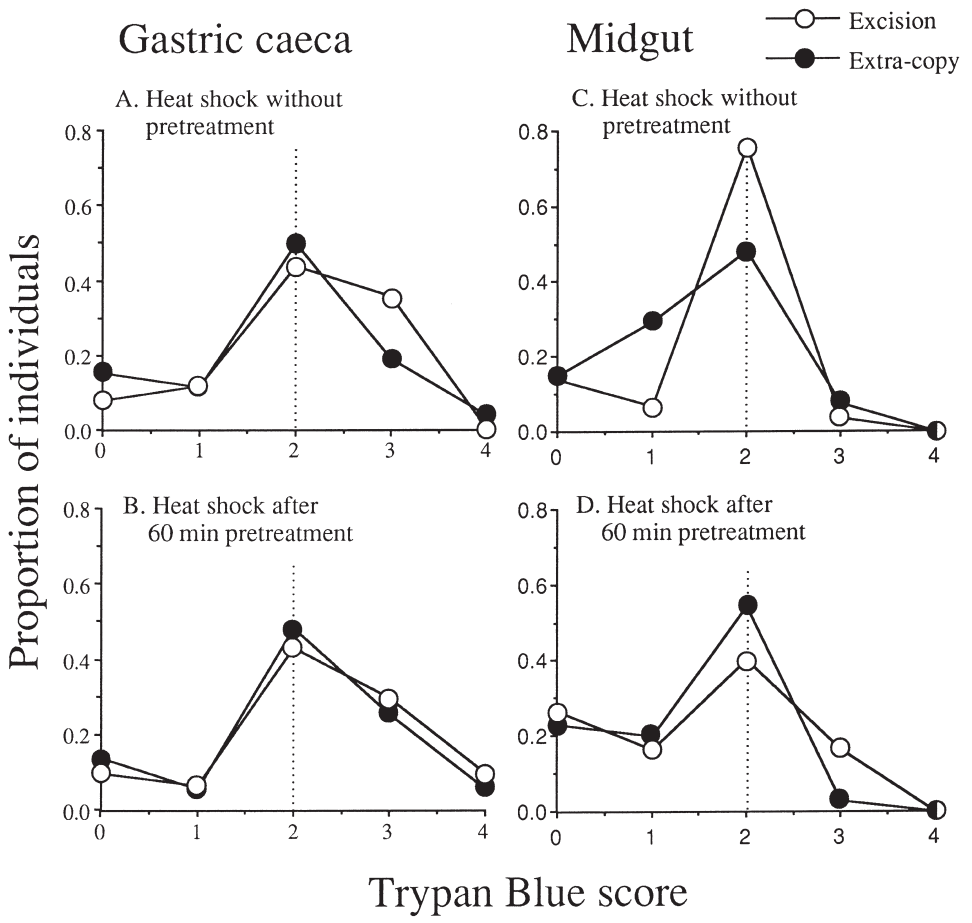

Fig. 5. Quantificatio of Trypan Blue staining within 2 h of heat shock in gastric caeca (A, B) and midgut (C,D) of 3rd-instar extra-copy (fille circles) and excision larvae (open circles). Pretreatment was at 36°C followed by a 60 min recovery period at 25°C, and heat shock was 60 min at 38.5°C. Scoring of tissues was: (0) no color; (1) any blue; (2) darkly stained nuclei; (3) large patches of darkly stained cells; (4) complete staining of most cells in the tissue. The number of individuals scored was for A and C, 29 excision and 27 extra-copy larvae and for B and D, 30 excision and 35 extra-copy larvae.

By 21 h after heat shock, staining increased in the caeca and midgut (comparing Fig. 5A, Fig. 5C and Fig. 6A, Fig. 6D, $P < 0.01$ for both tissues and strains) in larvae lacking pretreatment. Other tissues showed little increase in staining (Table 1), and the hindgut stained much less 21 h after heat shock than immediately after heat shock ($P < 0.01$ for both the excision and extra-copy strains). The excision and extra-copy larvae stained similarly in the absence of pretreatment.

By contrast, pretreated larvae stained less 21 h after heat shock than immediately afterwards, and the largest responses occurred in caeca and midgut (Fig. 6). After the long pretreatment, several tissues stained less than if not pretreated: caeca ($P < 0.001$, both strains), midgut (excision strain, $P < 0.001$; extra-copy strain, $P < 0.1$), and to a lesser extent, Malpighian tubules (Table 1). The short pretreatment likewise reduced staining in caeca of both strains ($P < 0.001$ in both strains) and in midgut of the extra-copy strain ($P < 0.001$). Midguts of excision larvae benefite little from a short pretreatment and, therefore, stained more than those of extra-copy larvae after a short pretreatment and heat shock (Fig. 6E, $P < 0.05$).

4. Discussion

In nature, *Drosophila* larvae inhabit necrotic fruit. Particularly within sun-exposed fruit, temperatures may rise sufficientl to induce expression of Hsp70 and/or to kill larvae (Feder et al., 1997). Because fruit temperatures may increase rapidly (Feder et al., 1997), the rate at which larvae become thermotolerant affects their probability to survive natural thermal stress.

Laboratory manipulation can quantify how the level of Hsp70 affects acclimation and survival. Under controlled conditions, inducible thermotolerance correlates closely with concentrations of Hsp70 (Fig. 7). At least some component of inducible thermotolerance is due to changes in Hsp70 levels, either directly through their effect on denatured proteins or by an interaction with other cellular components, because the extra-copy and

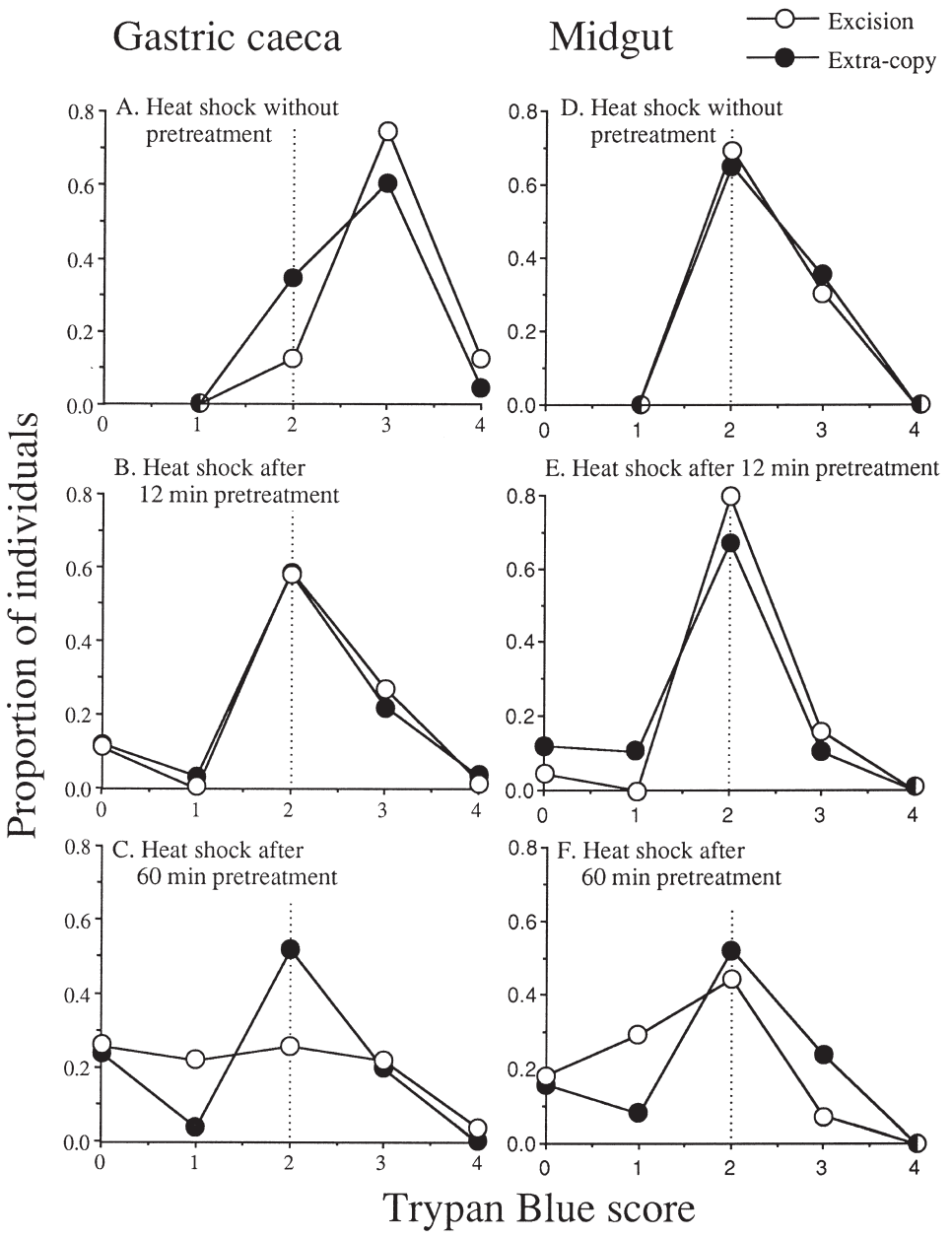

Fig. 6. Quantificatio of Trypan Blue staining 21 h after heat shock in gastric caeca (A–C) and midgut (D–F) of 3rd-instar extra-copy (fille circles) and excision larvae (open circles). Pretreatment was at 36°C followed by a 60 min recovery period at 25°C, and heat shock was 60 min at 38.5°C. Scoring of tissues was: (0) no color; (1) any blue; (2) darkly stained nuclei; (3) large patches of darkly stained cells; (4) complete staining of most cells in the tissue. The number of individuals scored was for A and D, 33 excision and 43 extra-copy larvae, B and E, 44 excision and 58 extra-copy larvae, and C and F, 27 excision and 25 extra-copy larvae.

excision strains vary primarily in this one protein and in inducible thermotolerance (Welte et al., 1993). Indeed, denatured proteins alone may induce Hsp expression (Wolfe et al., 1986). In both strains, larval thermotolerance increased rapidly after pretreatment, but shorter pretreatments increased thermotolerance more in the extra-copy strain, the strain that produced Hsp70 faster (Feder et al., 1996). A low level induction of Hsp70, such as that induced by a short or low intensity pretreatment, produced a disproportionate increase in thermotolerance. Long and intense pretreatments induced higher concentrations of Hsp70 but improved survival only marginally more than did a short pretreatment. Thus the relationship between changes in Hsp70 and thermotolerance was not linear. In any event, even relatively brief or mild pretreatments, which larvae may frequently encounter in necrotic fruit in nature, are clearly sufficien to induce both Hsp70 expression (Feder et al., 1997; present study) and thermotolerance. Whether these inducible events affect fitnes in nature depend upon any ensuing heat shock, which can match the induced thermotolerance or be so severe that no tolerance mechanism

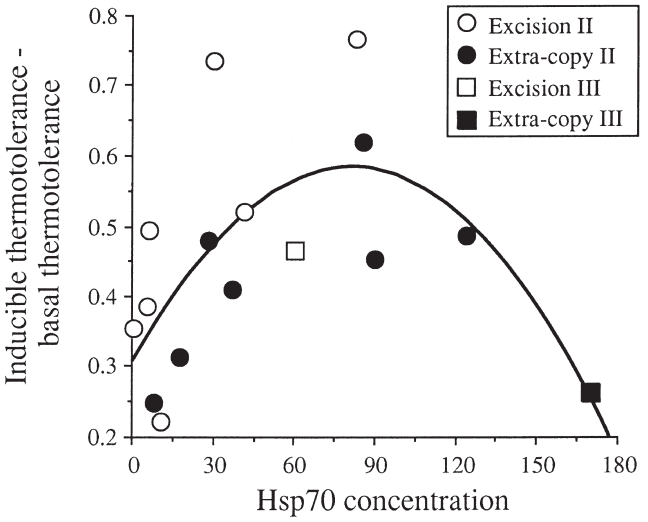

Fig. 7. Effect of Hsp70 level before heat shock on the induced increase in thermotolerance above basal levels in 1st-instar larvae, which was quantifie by the proportion of pretreated larvae surviving heat shock minus the survival proportion of unpretreated larvae. Hsp70 level before heat shock was manipulated by exposing engineered extra-copy and excision-control larvae to pretreatments varying in duration (10–30 min at 36°) and intensity (32°–36°C for 60 min). Hsp70 is expressed as a percentage of a standard. Heat shock varied among strains (39.2°C in the chromosome II strains utilized in this study, and 38.5°C in the chromosome III strains used in Krebs and Feder, 1997b), but both treatments resulted in approximately 15% survival in unpretreated larvae. The data were fitte to a polynomial regression (± 1 SE): The increase in tolerance = 0.31 ± 0.06 + (0.007 ± 0.002 × Hsp70)–(0.00004 ± 0.00001 × $Hsp70^2$), $R^2 = 0.45$, $P = 0.02$; linear regression is not significan ($R^2 = 0.02$; $P = 0.57$).

will ensure survival (Krebs and Loeschcke, 1994; Feder et al., 1997; Feder, 1997).

The cause of greater proportional benefit at low concentrations may be that Hsp70 protects its own expression at potentially lethal temperatures. Above 38°C, non-pretreated individuals either do not express Hsp70 or express Hsp70 very slowly (Krebs and Feder, 1997c), but once cells produce some Hsp70, additional expression ensues. Therefore, Hsp70 concentration may vary more after pretreatment alone than after pretreatment and heat shock, reducing differences in thermotolerance at intermediate concentrations of Hsp70 (Fig. 7).

The temporal patterns of Hsp70 expression provide insight to the mechanisms responsible for benefit in thermotolerance. One pattern is the rapid response to stress in some but not all tissues of *D. melanogaster* (Krebs and Feder, 1997c). Heat stress damages the larval gut more than almost any other tissue, and this damage may kill larvae slowly, potentially from desiccation or loss of nutrients (Krebs and Feder, 1997c). Pretreatment failed to block Trypan Blue staining in midgut, but it facilitated recovery from this heat damage. Pretreatment also reduced the chronic levels of Hsp70 in midgut, which may indicate lesser concentrations of denatured proteins (Hofmann and Somero, 1995). This relationship between pretreatment duration and repair (i.e. diminution in both Hsp70 level and Trypan Blue staining) suggests that Hsp70 contributes to repair of cell damage after heat shock. This, then, is consistent with a define role for Hsp70 in the protection of a specifi tissue against thermal damage, as is the preliminary findin that gut specifi expression of Hsp70 off of a heterologous promoter is sufficien to protect feeding against thermal inhibition in larval *Drosophila* (Feder and Krebs, 1997b). Extra *hsp70* copies reduced staining after a short pretreatment but provided no benefi either in the absence of pretreatment, where neither strain possessed Hsp70 before heat shock, or after an intense pretreatment, where the strains differed in Hsp70 due to expression above natural levels (Krebs and Feder, 1997b, and Krebs, Feder and Lee, unpublished) in the extra-copy strain. That benefit to organismal survival and midgut staining coincide only after a short pretreatment suggests that these phenotypes are linked, but, because high Hsp70 concentrations benefite survival much more than repair of this tissue, the predominant gain from extra Hsp70 may lie elsewhere in the larva.

While Hsp70 may provide benefit away from the gut, so too may costs from very high Hsp70 concentrations lie elsewhere. Too much Hsp70 has costs, even for thermotolerance (Fig. 7); after high concentrations of Hsp70 or constitutive expression, larval survival declines both in the presently analyzed strains and another extra-copy/excision pair (Krebs and Feder, 1997a), and cell growth slows (Feder et al., 1992). Overexpression increased neither Trypan Blue staining nor chronic Hsp70 expression in any tissue. Evidence on the timing of mortality in the extra-copy and excision lines suggests that larvae die more rapidly from overexpression of Hsp70 after pretreatment and heat shock than from direct heat damage (Krebs and Feder, 1997a).

Perhaps because variation in Hsp70 has diverse consequences, *D. melanogaster* tightly regulates its expression (Lindquist, 1993). Regulatory controls can evolve through trade-offs between positive and negative effects on cell and organismal function, as follows: Individuals should regulate expression to produce moderate (but not too high) concentrations of Hsp70 immediately after exposure to heat and other stresses, to eliminate expression in the absence of stress (Solomon et al., 1991), and to eliminate Hsp70 as soon as recovery is complete (DiDomenico et al., 1982a), but to prevent concentrations from exceeding a critical level. Hsp70 mRNA transcripts destabilize on return of *Drosophila* cells from stress conditions (DiDomenico et al., 1982b), which should reduce further Hsp70 synthesis. Benefit and costs of different regulatory patterns, however, may vary in different environments. Environments where stress is not common may favor individuals that produce Hsp70 slowly because they will rarely overexpress this protein or express it unnecessarily. Conversely, stressful

environments may favor individuals that express Hsp70 very rapidly, because they select for a more extreme response to heat. Hsp70 obviously cannot explain all of inducible stress tolerance. Induced thermotolerance may persist long after Hsp70 concentrations in *Drosophila* become undetectable (Krebs and Loeschcke, 1994, 1995; Krebs and Feder, 1997a). Increased concentrations of osmolytes are one of several alternative mechanism that provide thermotolerance (Clegg and Jackson, 1992). Nonetheless, raising Hsp70 concentration may be the most rapid response to thermal stress in *Drosophila*. Consequently, two aspects of the environment, the probability that a larva will encounter high temperature and the rate at which temperatures rise, may affect how Hsp70 expression evolves within natural populations.

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