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Evolution of thermotolerance and the heat-shock response: evidence from inter/intraspecific comparison and interspecific hybridization in the *virilis* species group of *Drosophila*. I. Thermal phenotype

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Summary

Species in the *virilis* group of *Drosophila* (fruit flies), which overlap or replace one another along climatic gradients, exhibit corresponding differences in basal thermotolerance, inducible thermotolerance and the heat-shock response. The low-latitude species *D. virilis* exceeds the high-latitude species *D. lummei* in these measures of thermotolerance, the temperature threshold for heat-shock factor (HSF) activation and the ability to express *hsp70* mRNA and diverse heat-shock proteins (e.g. Hsp70, Hsp83 and small Hsps) after intense heat shock (e.g. 40–41°C). The xeric species *D. novamexicana* differs from the mesic species *D. texana* in much the same way for many of these traits. By contrast, intraspecific variation in these traits is small. Because *D. virilis* and *D. lummei* can readily be crossed to yield partially fertile progeny, genetic analysis of interspecific differences is possible. Interspecific hybrids are intermediate to the parental species in basal thermotolerance and inducible thermotolerance and resemble *D. virilis* in Hsp concentrations after intense heat shock and Hsp70 protein electromorphs.

Key words: *Drosophila*, evolutionary physiology, heat-shock protein, Hsp70, molecular chaperone, countergradient variation.

Introduction

Investigations in evolutionary physiology normally fall into two separate realms. The first concerns the large differences in phenomes among species or higher taxa, whereas the second concerns the often lesser variation within species (Feder et al., 2000). These realms are ordinarily distinct because of reproductive isolation among species. Thus, because species and higher taxa usually cannot be interbred experimentally, investigation of their physiological differences often must proceed in the absence of experimental manipulative genetics. Genetic studies within species, however, often must forego the larger physiological variation that interspecific studies offer. Here, we report on variation in thermotolerance and the heat-shock response in a remarkable instance in which these two normally separate realms of investigation coalesce. The *virilis* group of *Drosophila* comprises 12 species living in greatly differing habitats but amenable to interbreeding in the laboratory (Patterson and Stone, 1952; Throckmorton, 1982). We have exploited these attributes to investigate the genetic basis of interspecific differences in physiology in combination with comparative inference.

The *virilis* group is divisible into two phylads: *virilis* and *montana* (Patterson and Stone, 1952). In the *virilis* phylad, two species, *D. virilis* and *D. lummei*, populate opposite ends of a climatic/geographic spectrum but overlap in its center. According to many investigators (Evgenev et al., 1982; Nurminsky et al., 1996; Patterson and Stone, 1952; Spicer, 1991, 1992; Throckmorton, 1982), *D. virilis* is the most primitive species of the *virilis* phylad and is probably ancestral to it if not to the entire *virilis* group. Its distribution, which extends across Eurasia, is primarily below 40° N latitude. *D. lummei*, considered the closest relative of *D. virilis*, occurs from just above 40° to just above 65° N latitude and from Sweden east to the Pacific coast of Asia. In the New World, *D. novamexicana* and *D. texana* are a similar but less widely distributed species pair from the *virilis* phylad. *D. novamexicana* occurs in hot, arid environments in New Mexico, Arizona, Colorado, Utah (Hsu, 1952) and elsewhere, whereas *D. texana* inhabits more mesic environments in central Texas.

If the microclimates that these species experience differ in the same ways as their climatic ranges, then data for many other species suggest that their thermal phenotypes (Feder, 1996) should differ correspondingly. Specifically, for organisms in general (Cossins and Bowler, 1987), and *Drosophila* in particular (David et al., 1983; Feder and Krebs, 1998; Hoffmann et al., 2003; Stratman and Markow, 1998), tolerance

of high temperatures and its underlying mechanisms are correlated with the typical thermal environments of species (i.e. countergradient variation). From this prior work, we focus on several discrete aspects: basal thermotolerance (tolerance of acute exposure to hyperthermia in naive organisms or cells), inducible thermotolerance [the change in thermotolerance when mild hyperthermia (= pre-treatment; PT) precedes exposure to more-severe hyperthermia] and the heat-shock response. The heat-shock response comprises the PT-inducible expression of heat-shock proteins (Hsps), molecular chaperones and other proteins that contribute to inducible thermotolerance (Feder and Hofmann, 1999; Ulmasov et al., 1992; Zatsepina et al., 2001). The most important of these in other *Drosophila* species is Hsp70, a member of the DnaK-Hsp70 superfamily (Feder and Krebs, 1998; Zatsepina et al., 2001). Prior work on these aspects in the *virilis* group, however, is limited. *D. virilis* exceeds *D. lummei* in basal thermotolerance (Garbuz et al., 2002; Mitrofanov and Blanter, 1975), as expected. In the cold, *D. lummei* is far more tolerant than *D. virilis*, as expected, and also undergoes a diapause absent in all other *D. virilis* group species (Lumme, 1982). Heat-inducible loci have been localized cytogenetically in *D. virilis* (Evgenev et al., 1978; Peters et al., 1980). Although Sinibaldi and Storti (1982) reported that the two species do not differ in Hsps induced by heat shock, Garbuz et al. (2002) have recently shown that both the *D. virilis–D. lummei* and *D. novamexicana–D. texana* species pairs differ in Hsp induction. In both cases, the former member of each pair synthesizes greater amounts of Hsps after heat shock. The present study extends the work of Garbuz et al. (2002) by comparing additional strains of *D. virilis* and *D. lummei*, focusing on more-intense heat shock and examining *hsp* mRNA expression and its regulation. Heat-inducible loci have been localized cytogenetically in *D. virilis* (Evgenev et al., 1978; Peters et al., 1980).

Ordinarily, understanding the evolution of such interspecific variation would be amenable only to comparative inference and inaccessible to genetic experimentation. In the 1970s, however, Evgenev and colleagues were able to cross *D. lummei* with a *D. virilis* strain (160) bearing recessive markers on all autosomes and subsequently developed strains in which a single *lummei* chromosome or portion thereof was integrated into a uniform *D. virilis* background (Evgenev and Sidorova, 1976). In the present study, we combine comparative inference and interspecific genetics to elucidate the molecular basis for interspecific variation in thermotolerance. We demonstrate (1) replicated countergradient variation of basal thermotolerance in two geographically distinct sets of *virilis* phylad species; (2) countergradient variation in inducible thermotolerance and the heat-shock response in *D. virilis* and *D. lummei*; and (3) the applicability of interspecific genetics to inducible thermotolerance (and prospectively other aspects of the thermal phenotype) in the *virilis* species group of *Drosophila*.

Materials and methods

Drosophila *strains and maintenance*

We compared laboratory strains of four species from the *virilis* phylad and one reference strain of *Drosophila melanogaster* (Table 1). All strains were maintained at 25°C on a yeast, molasses and agar medium for many generations before analysis. Hybrids of *D. virilis* strain 9 and *D. lummei* strain 200 were created by mass mating of heterospecific parents of the opposite sex.

Thermotolerance

These procedures were identical to those of Garbuz et al. (2002). Eclosing individuals were sequestered daily and, when 4 days old, were transferred by aspiration to a preheated

Table 1. Drosophila *strains examined in the present study*

Species	Strain	Origin	LT_{50} (°C)	Latitude of collection site; comments
D. virilis Sturtevant	T53	Tashkent	41.2	41° N; especially warm climate
	T61	Tashkent	41.1	41° N; especially warm climate
	9	Batumi (Georgia)	41.3	42° N; caucasus; not as warm as Tashkent
	160	Japan	40.8	~32–36° N; marker strain with many mutations
	101	Japan	41.2	~32–36° N; wild-type strain
	1433	Leeds, UK	41.1	53° N
D. lummei Hackman	202	South Russia	40.1	45° N
	207	South Russia	40.2	45° N
	200	Moscow	40.1	55° N
	1102	Finland	40.2	60° N
	1109	Finland	40.2	60° N
D. texana Patterson	423	TX, USA	41	~32° N; woodland
D. novamexicana Patterson	424	NE, USA	42	~32–37° N; desert
D. melanogaster Meigen	Oregon R	OR, USA	40	43° N; collected in 1925

LT_{50}, a measure of basal thermotolerance, is the temperature of a 30-min heat shock resulting in 50% mortality, as interpolated from the data of Fig. 1. Temperatures resulting in >99% mortality are given in Garbuz et al. (2002).

polypropylene vial (Nalge Nunc International, Rochester, NY, USA), which was immersed in a thermostatted water bath for 30 min. Each vial usually contained 35–50 animals but occasionally as few as 20. To determine basal thermotolerance, vials were placed at one of a series of heat-shock temperatures ranging from 38.5°C to 43°C. To determine inducible thermotolerance, similar determinations ensued after flies first underwent pre-treatment at one of a series of temperatures from 35°C to 38°C for 30 min and 25°C for 1 h or 3 h. Tolerance was assessed as the proportion of flies in a vial that could walk 48 h after heat shock.

Protein labeling, gel electrophoresis and immunoblotting

These procedures were identical to those of Garbuz et al. (2002). 10 salivary glands from third-instar larvae were labeled in 20 µl of Schneider's insect medium without methionine (Sigma, St Louis, MO, USA) after the addition of 1 µl (1.85 MBq) of [^{35}S]L-methionine (Amersham Biosciences Corp., Piscataway, NJ, USA) for 1 h at 25°C after various treatments. Two-dimensional gel electrophoresis and other procedures applied were as described (O'Farrell et al., 1977; Ulmasov et al., 1992). The position of major Hsps and actin was determined by both autoradiography and subsequent staining of gels with silver (Creighton, 1990).

For immunoblotting, after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of larval lysate prepared as above, the proteins were transferred to nitrocellulose membrane (Hybond ECL; Amersham) according to the manufacturer's protocol and reacted with monoclonal antibodies specific to the entire *Drosophila* Hsp70 family (7.10.3) and only Hsp70 (7FB) as previously described (Zatsepina et al., 2001). Immune complexes were detected *via* chemiluminescence (ECL kit; Amersham) and diaminobenzidine (DAB) (Sigma) with appropriate peroxidase-conjugated anti-rat secondary antibodies.

Preparation of RNA and northern hybridization

RNA was prepared by the standard method with 4 mol l^{-1} guanidine isothiocyanate (Chomczynski and Sacchi, 1987), separated by agarose gel electrophoresis and transferred to a membrane for hybridization (Sambrook and Fritsch, 1989) with a *Cla*I–*Bam*HI fragment containing the *Drosophila melanogaster hsp70* gene cloned into the *Bam*HI site of pUC13 (McGarry and Lindquist, 1985). Hybridization was overnight at 42°C in 50% formamide, followed by two 20 min washes in 2×SSC, 0.2% SDS at 42°C, two 20 min washes in 1×SSC, 0.2% SDS at 42°C, and one 20 min wash in 0.2×SSC, 0.2% SDS at 68°C.

Gel mobility-shift assay

Flies were frozen and pulverized in liquid nitrogen, and the powder was suspended (1:5) in a buffer containing 20 mmol l^{-1} Hepes, pH 7.9, 25% (v/v) glycerol, 0.42 mol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 0.5 mmol l^{-1} dithiothreitol, which was centrifuged at 100 000 ***g*** for 20 min. The supernatants were frozen in liquid nitrogen and stored at –70°C. The protein concentration of the extracts was estimated with a modified Lowry method (Ulmasov et al., 1992).

Consensus HSE probe (Wu et al., 1988) was prepared by annealing partially complementary oligonucleotides (ATCCG-AGCGCGCCTCGAATGTTCTAGAA and CTCGCGCGG-AGCTTACAAGATCTTTTCCA) in 10 mmol l^{-1} potassium phosphate buffer, pH 8.2, in the presence of 0.1 mmol l^{-1} NaCl. Single-stranded termini were filled with Klenow polymerase and [^{32}P]ATP (Sambrook and Fritsch, 1989). For the gel mobility-shift assay, extracts containing 50 µg of protein were mixed with 0.5 ng of [^{32}P] heat shock element (HSE) in binding buffer as described (Mosser et al., 1993). Binding-reaction mixture was incubated at room temperature (20°C) for 20 min. Free probe was separated from HSE–HSF complexes by electrophoresis in 5% polyacrylamide gels (Mosser et al., 1993). The gels were dried and exposed to X-ray film (Kodak X-Omat) at –70°C.

Results

Thermotolerance

The basal thermotolerance of adult flies of all *D. virilis* strains used in the study exceeded that for its sister species *D. lummei* (Fig. 1; Table 1). The LT_{50}s for a 30-min heat shock are generally 1.3°C greater for *D. virilis* strains than for *D. lummei* and are inversely related to their latitude of origin. Similarly, basal thermotolerance for *D. novamexicana* exceeded that for its related species *D. texana*. Although both are from similar latitudes, *D. novamexicana* is from a more arid and warmer climate than *D. texana* (Table 1).

Within *D. virilis* and *D. lummei*, we failed to detect differences as large as those among species. This minor intraspecific variation in basal thermotolerance, moreover, was not inversely correlated with latitude. With the exception of strain 160, the LT_{50} for *D. virilis* strains ranging from 32° to 53° N were within 0.2°C of one another and, with the exception of strain 1102, the LT_{50} for *D. lummei* strains ranging from 45° to 60° N were within 0.1°C of one another. Strain 160 is a marker strain with at least one known recessive mutation on each autosome (broken *bk* in ch. II; gapped *gp* in ch. III; cardinal *cd* in ch. IV; peach *pe* in ch.V; and glossy *gl* in ch. VI); all other strains are wild-type.

Reciprocal hybrids of *D. virilis* and *D. lummei* had basal thermotolerances intermediate to those of the two parental species (Fig. 2A). The direction of the cross did not affect basal thermotolerance appreciably.

In *D. virilis* strain 9, pre-treatment improved thermotolerance (Fig. 2B), and the change in thermotolerance (i.e. LT_{50} after PT minus LT_{50} without PT) was correlated with the PT temperature. The impact of PT on thermotolerance in *D. virilis* strain 160 was nearly identical to that in strain 9, although the absolute values of LT_{50}s differed in these two strains (Fig. 1; data after PT not shown for strain 9). By contrast, pre-treatment reduced thermotolerance in *D. lummei*, and the change in thermotolerance was inversely correlated

with the PT temperature (Fig. 2B). PT at 36°C and 37°C reduced survival of 40–40.5°C heat shock to 0%.

A *D. virilis* parent, regardless of sex, was sufficient for positive inducible thermotolerance in *D. virilis–D. lummei* hybrids (Fig. 2B). Although results differed slightly according to the direction of the cross, PT temperature and the interval between PT and heat shock, PT increased the LT_{50} of *D. virilis–D. lummei* hybrids by 0.25–0.5°C, to where it was slightly less than the basal thermotolerance of pure *D. virilis*. Although this PT was modest, it clearly differs from the decreased thermotolerance after PT in *D. lummei*. In the hybrids, the effect of increasing PT temperature was intermediate to that in the parental species; it neither increased nor decreased inducible thermotolerance.

Protein synthesis and levels

As Garbuz et al. (2002; figs 5, 6) have previously reported, under normal physiological conditions (25°C), *D. virilis* strain 9 and *D. lummei* strains 200 and 1102 did not differ in total protein synthesis, as indicated by [^{35}S]L-methionine incorporation. At 1 h after mild heat shock (37.5°C), these *D. lummei* strains synthesized no less protein than did *D. virilis* strain 9. By contrast, at 1 h after more-intense heat shock (40°C), these *D. lummei* strains clearly synthesized less protein than did *D. virilis* strain 9. These differences are manifest in all major classes of heat-shock proteins that are typically distinguishable in one-dimensional electrophoresis of synthesized proteins. Additional determinations of protein synthesis *via* [^{35}S]L-methionine incorporation (Figs 3–4) corroborate and extend these conclusions. First, as inclusion of two additional *D. virilis* strains (160 and 1433) demonstrates, these are true interspecific differences rather than a feature unique to *D. virilis* strain 9. Second, these interspecific differences are even greater after more-intense heat shock (40.5°C and 41°C), although both species are capable of some protein synthesis at all temperatures studied. After these intense heat shocks, Hsp40 and small Hsps are especially reduced in *D. lummei* relative to *D. virilis*. Third, interspecific hybrids (*D. lummei* strain 200 × *D. virilis* strain 9) exhibit the *D. virilis* pattern of protein synthesis. The same is true of the reciprocal cross (data not shown). Finally, the species pair *D. novamexicana* and *D. texana*, which correspond in thermotolerance to *D. virilis* and *D. lummei*, respectively, exhibit parallel differences in protein synthesis (Fig. 4). Interestingly, *D. virilis* strain 160, which both has numerous mutations and is the most thermosensitive of the *D. virilis* strains (see above), is not obviously defective in terms of protein synthesis after heat shock. Also, both *D. lummei* and *D. virilis* exhibit expression of a high-molecular-mass heat-shock protein under some conditions (Fig. 3).

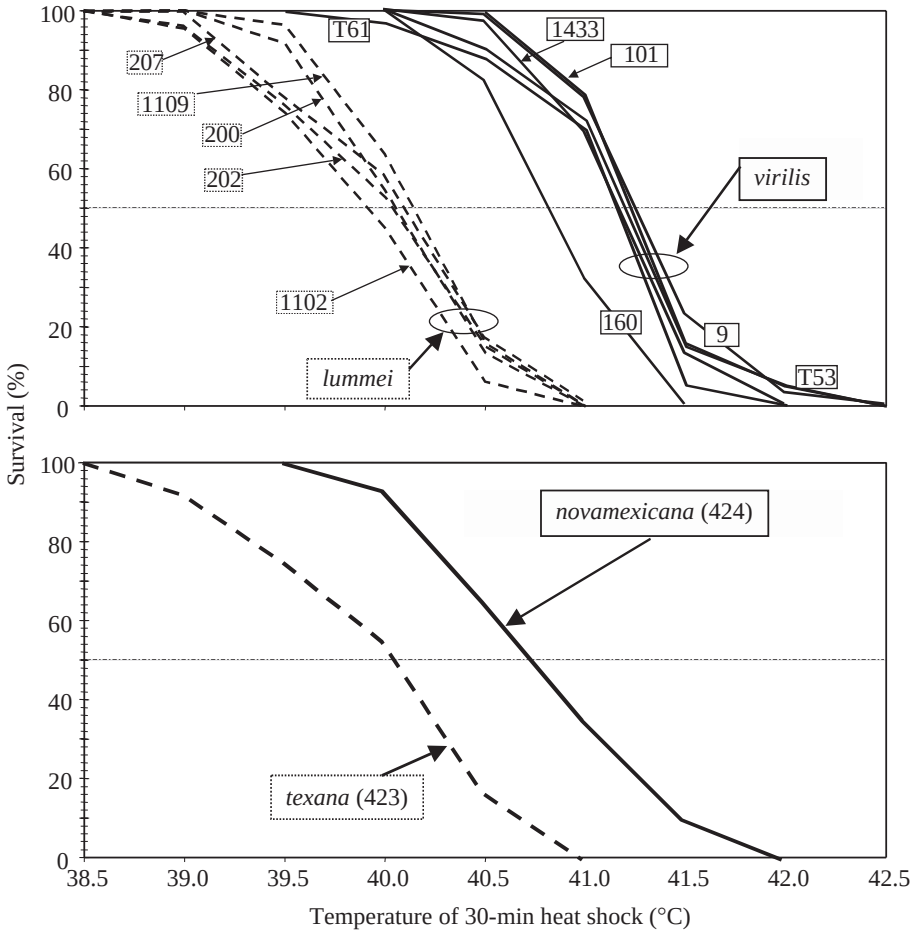

Fig. 1. Inter- and intraspecific variation of basal thermotolerance in *D. virilis* group species. Strain 160, although *D. virilis*, is a marker strain with at least one known recessive mutation on each chromosome; all other strains are wild-type. See Table 1 for additional descriptions of all strains. Data for *D. lummei* strain 200 and *D. virilis* strains 9, 160 and 1433 are replotted from Garbuz et al. (2002), in which *D. virilis* strain 1433 is mistakenly labeled strain 1590.

According to both Garbuz et al. (2002) and the present study (Figs 3, 4), Hsp70 is quantitatively the major heat-shock protein in the species and strains examined. To examine the conclusions of the previous paragraph in detail for this specific Hsp, we have replotted the data from Fig. 2A of Garbuz et al. (2002) and included data for additional strains and conditions (Fig. 5). Indeed, Hsp70 levels for *D. lummei* strains are within the range for the various *D. virilis* strains 3 h after a 37.5°C heat shock. After more-severe heat shock, Hsp70 levels in the *D. lummei* strains are below the range for *D. virilis* strains, corresponding to their differing thermotolerances (Figs 1, 2). Data for the more-thermotolerant *D. novamexicana* and less-thermotolerant

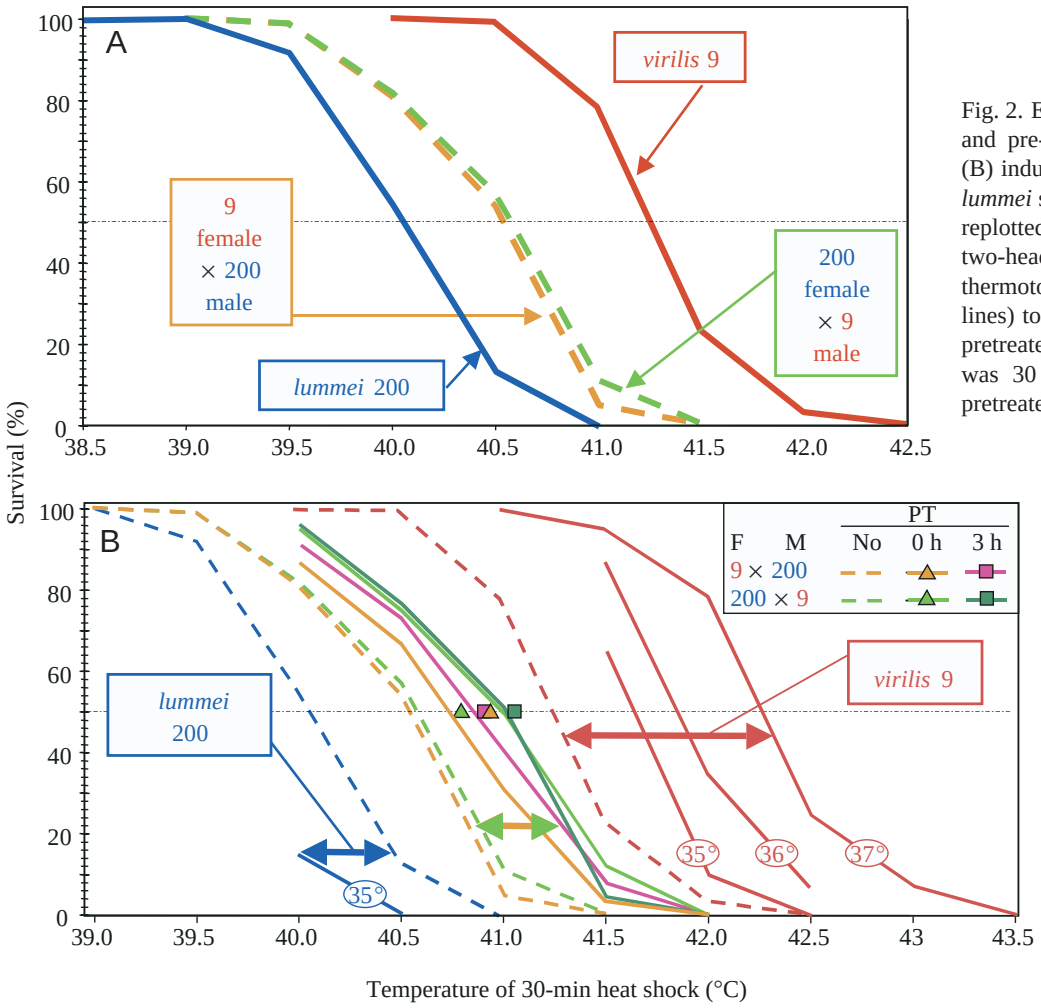

Fig. 2. Effect of species, parental genotype and pre-treatment (PT) on (A) basal and (B) inducible thermotolerance. Data for *D. lummei* strain 200 and *D. virilis* strain 9 are replotted from Garbuz et al. (2002). In B, two-headed arrows represent the change in thermotolerance from basal levels (broken lines) to tolerance after PT (solid lines). In pretreated *D. virilis* strain 9 (solid red), PT was 30 min at 35°C, 36°C or 37°C. In pretreated *D. lummei* strain 200 (solid blue), PT was 30 min at 35°C (as shown), 36°C (not shown) or 37°C (not shown). No *D. lummei* pre-treated at 36°C or 37°C survived the ensuing heat shock. The inset indicates color coding for reciprocal hybrids of *D. lummei* strain 200 and *D. virilis* strain 9 and the species identity of female (F) and male (M) parent. Broken and solid lines indicate thermotolerance with and without pretreatment, respectively, after 35°C PT for 30 min. Hybrids also underwent PT at 36°C for 30 min with heat shock either following immediately (triangles) or 3 h later (squares); these symbols signify the LT_{50}s.

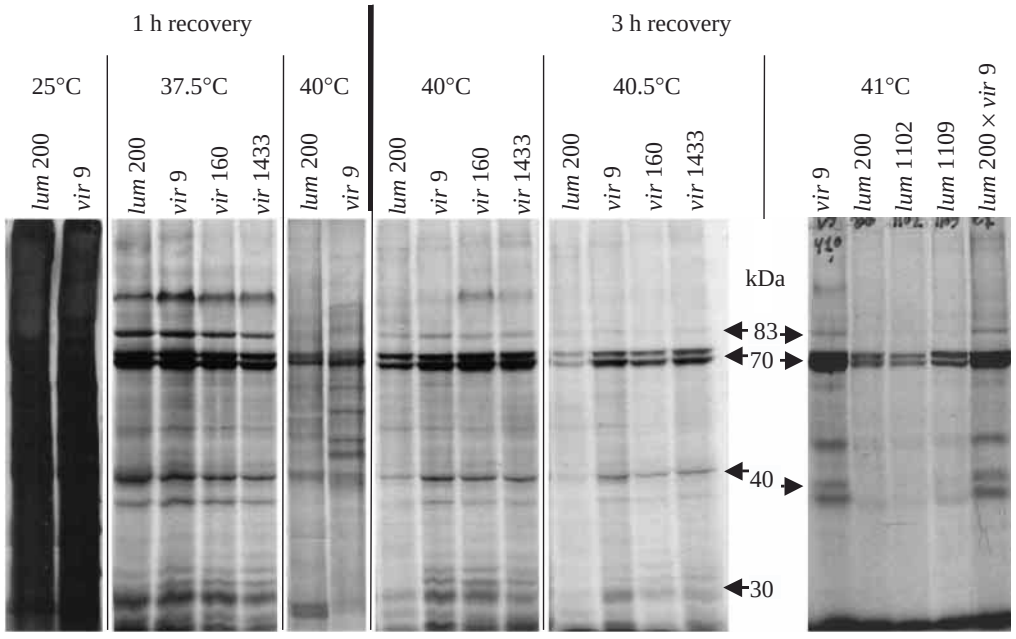

Fig. 3. One-dimensional electrophoretic separation of ^{35}S-labeled proteins after 30-min heat shock. *vir* refers to *D. virilis*, and *lum* refers to *D. lummei*; numbers represent strains. Size markers refer to expected molecular masses of *Drosophila* heat-shock proteins.

D. texana recapitulate this pattern (Fig. 5B). Immunoblots of Hsp70 levels (Fig. 5C) clearly emphasize the differing Hsp70 levels in these species after intense heat shock. Detailed examination of the Hsp70 family reveals that the differences between Hsp70s of *D. virilis* and *D. lummei* are qualitative as well as quantitative (Fig. 6; see also Garbuz et al., 2002). *D. virilis* exhibits three isoforms recognizable by antibody 7FB, which in *D. melanogaster* reacts only with Hsp70; *D. lummei*

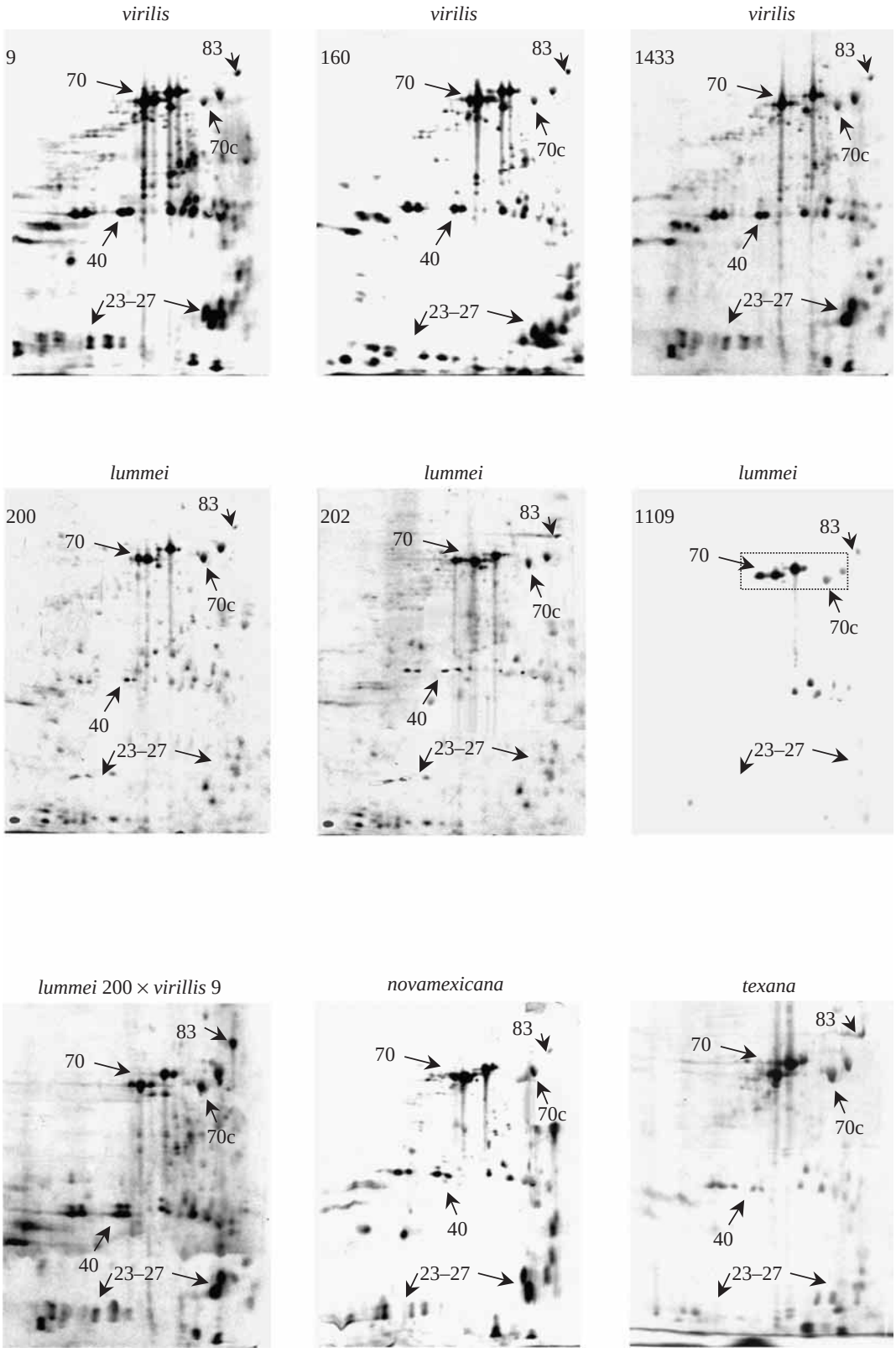

Fig. 4. Two-dimensional electrophoretic separation of ^{35}S-labeled proteins after 30-min heat shock at 40.5°C and 3 h of recovery at 25°C. Species and strains are noted. Labels refer to molecular masses of *Drosophila* heat-shock proteins; 70c represents constitutively expressed Hsp70 family members. The box in the Hsp70 region for *D. lummei* strain 1109 represents the region detailed in Fig. 6. See Garbuz et al. (2002) for additional results after less-severe heat shock.

exhibits only two of these three isoforms. *D. virilis* also exhibits two inducible isoforms not recognized by 7FB but recognizable by antibody 7.10.3, which reacts with all Hsp70 family members in most species examined; *D. lummei* exhibits only one of these two isoforms. Interspecific hybrids (*D. lummei* strain 200 × *D. virilis* strain 9) exhibit the *D. virilis* pattern (Fig. 6), as does the reciprocal cross (data not shown).

Transcriptional regulation

The thermal sensitivity of *hsp70* mRNA transcription in general resembled that for Hsp70 protein. Thus, *D. lummei* synthesized no detectable *hsp70* mRNA during heat shock at 40–41°C, whereas *D. virilis* strain 9 did so in abundance (Fig. 7). These results for *D. virilis* strain 9 exemplify those obtained for other *D. virilis* strains. Despite its low thermotolerance (Fig. 1) and unexceptional Hsp70 protein levels (Fig. 3), *D. virilis* strain 160 typically synthesized more *hsp70* mRNA than did other *D. virilis* strains (Fig. 7; and other data not shown).

As indicated by electrophoretic mobility-shift assays (Fig. 8), *D. virilis* underwent HSF trimerization at temperatures of >32°C, whereas *D. lummei* exhibited activation at temperatures of >31°C. This difference, although small, is again consistent with the environmental regimes of these species.

Discussion

Many aspects of the thermal phenotypes of the species under study exhibit countergradient variation similar to that in other taxa (Feder and Hofmann, 1999; Ulmasov et al., 1992; Zatsepina et al., 2001); i.e. the magnitude and threshold for traits correspond to the thermal environments in which the species occur. Thus, the lower-latitude species *D. virilis* exceeds the higher-latitude species *D. lummei* in basal thermotolerance (see also Garbuz et al., 2002), inducible thermotolerance and temperature threshold for HSF activation. Similarly, the more xeric *D. novamexicana* exceeds the more mesic *D. texana* in the former two traits. As also shown previously for protein (Garbuz et al., 2002), *D. virilis* and *D. lummei* express similar amounts of heat-shock mRNA and protein at low to moderate heat-shock temperatures. Intense heat shock (e.g. 40–41°C) almost abolishes heat-shock mRNA and protein expression in *D. lummei*, whereas considerable expression persists after such heat shock in *D. virilis*. For example, Hsp70 concentration is similar in *D. virilis* and *D. lummei* after 37.5°C heat shock but is markedly greater in *D. virilis* than in *D. lummei* after more-intense heat shocks (Fig. 5A); a similar pattern is evident for *D. novamexicana* and *D. texana* (Fig. 5B). In *D. lummei*, moreover, the reduction in protein level after severe heat shock is even greater for the small Hsps and Hsp40 than for Hsp70 (Figs 3, 4).

Within *D. virilis* and *D. lummei*, by contrast, little such countergradient variation is evident. We speculate that extensive gene flow among populations swamps incipient adaptation to local conditions, a situation that may not be universal in

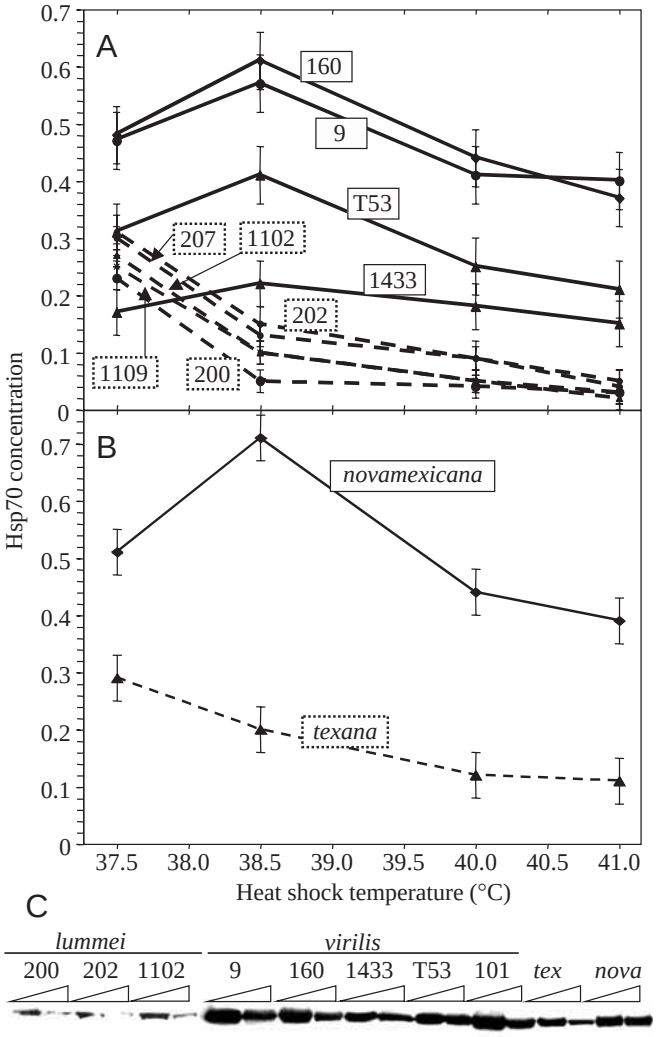

Fig. 5. Variation in Hsp70 concentration in salivary glands due to species, strain and heat-shock conditions. All determinations are for equivalent amounts of total protein extracted 3 h after a 30-min heat shock and are from immunoblots with 7FB, an antibody recognizing only the 70-kDa inducible Hsp70 family member in *D. melanogaster* (see Materials and methods). (A) Effect of heat-shock temperature on Hsp70 levels in *D. virilis* strains (solid lines) and *D. lummei* strains (broken lines). Symbols represent the mean densitometric signal ± 1 S.E.M. from at least five independent immunoblots. All data are normalized to the densitometric signal for equivalent amounts of protein extracted from *D. melanogaster* salivary glands 1 h after a 30-min heat shock at 37.5°C. (B) Effect of heat-shock temperature on Hsp70 levels in *D. novamexicana* (solid line) and *D. texana* (broken line). Data are standardized and plotted as for A. (C) 7FB-immunoblots of Hsp70 for various species and strains. Each pair of lanes corresponds to 40°C and 41°C heat shock. See fig. 2 in Garbuz et al. (2002) for additional determinations of Hsp70 levels.

Drosophila species (Michalak et al., 2001). As in *D. melanogaster* (Krebs et al., 2001), adaptation to laboratory conditions does not seem to contribute to this intraspecific similarity, as the two strains from Tashkent, Uzbekistan (T53 and T61) exhibit virtually identical patterns of thermotolerance

and Hsp induction despite the fact that T61 was captured recently and the T53 more than 30 years ago. The only conspicuous departure from this pattern is for *D. virilis* strain 160, a marker strain with at least one known recessive mutation on each autosome. The basal thermotolerance for this strain is considerably lower than for all other *D. virilis* strains studied, suggesting that the mutations it bears interfere with this form of thermotolerance. Its expression of heat-shock proteins, which underlies inducible rather than basal thermotolerance, is as in other *D. virilis* strains, however.

The absence of inducible thermotolerance in the high-latitude species *D. lummei* is indeed distinctive. We know of no other *Drosophila* species that shares this absence. Remarkably, this species expresses a full complement of heat-shock proteins after heat pre-treatment (although in lesser magnitude than in *D. virilis*), suggesting that these proteins are not sufficient for inducible thermotolerance and that some unknown component is deficient.

An unusual feature of the *virilis* species group is its capacity for interspecific introgression of genetic material in the laboratory. Here, we demonstrate corresponding patterns in basal and inducible thermotolerance (Fig. 2), overall protein synthesis after heat shock (Figs 3, 4) and Hsp70 electromorphs (Fig. 6). For thermotolerance, the pattern for hybrids is intermediate to that for the two parental species and independent of the direction of the cross. For quantitative and qualitative variation in Hsp expression, the *D. virilis* pattern behaves as a dominant trait. For example, *D. virilis* exhibits three Hsp70-family protein isoforms recognizable by both antibody 7.10.3 and 7FB, *D. lummei* exhibits only two isoforms, and hybrids exhibit three isoforms (Fig. 6). Similarly, *D. virilis* exhibits two inducible Hsp70-family protein isoforms recognizable by antibody 7.10.3, *D. lummei* exhibits only one isoform, and hybrids exhibit two isoforms (Fig. 6). The *Drosophila virilis* group has great potential to elucidate the genetic basis for interspecific differences in thermal phenotype for two reasons. First, the group is far more diverse than the four species examined here;

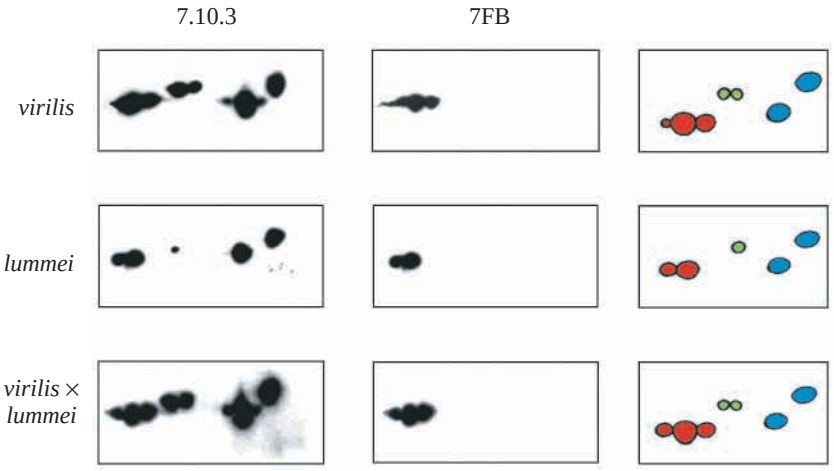

Fig. 6. Immunoblots of Hsp70 and Hsp70 family members in *D. virilis* strain 9, *D. lummei* strain 200 and their hybrid. In Fig. 4, the box in the Hsp70 region for *D. lummei* strain 1109 represents the region of the present figure. Data for the parental strains are repeated from Garbuz et al. (2002) and are included to facilitate comparison with data for hybrids. Primary antibody 7FB recognizes only the 70-kDa inducible Hsp70 family member in *D. melanogaster*; primary antibody 7.10.3 recognizes all Hsp70 family members (see Materials and methods). Heat shock was 38°C for 30 min, with 3 h recovery at 25°C before lysis. In the right-hand column, blue represents constitutively present proteins recognized by 7.10.3 only, red represents inducible (i.e. undetectable in glands not undergoing heat shock) proteins recognized by both 7.10.3 and 7FB, and green represents inducible proteins recognized by 7.10.3 only.

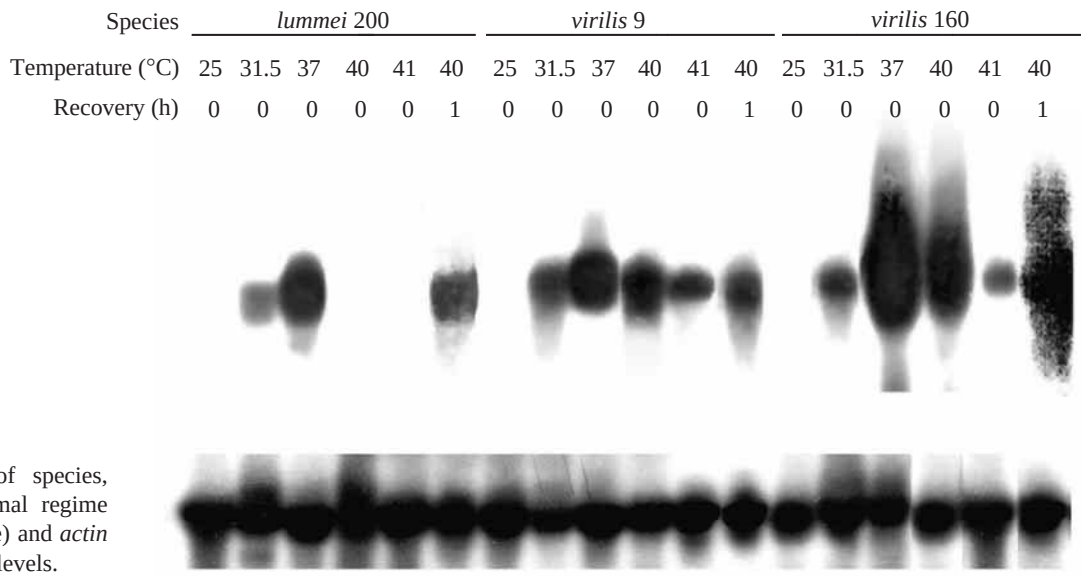

Fig. 7. Effect of species, strain and thermal regime on *hsp70* (above) and *actin* (below) mRNA levels.

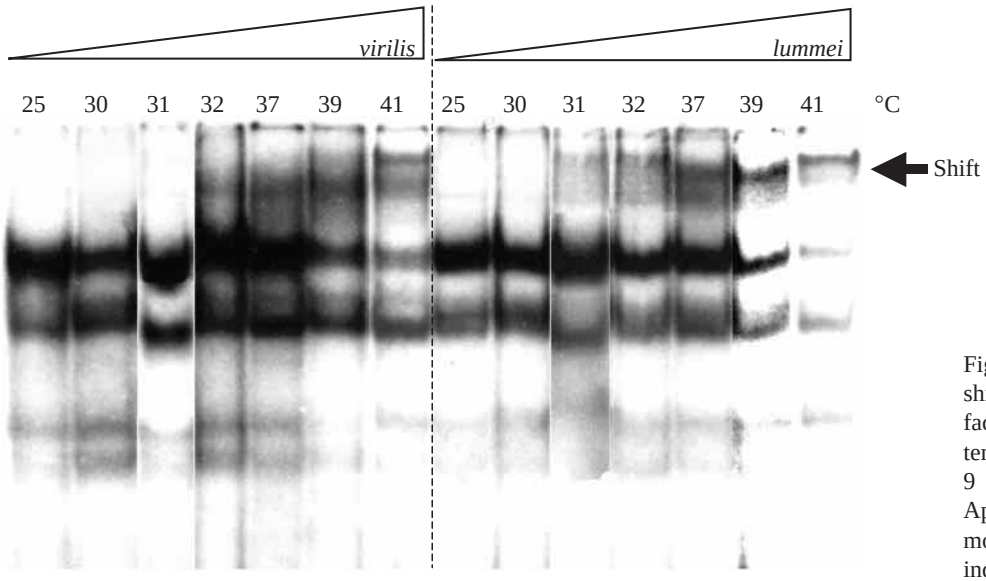

Fig. 8. Electrophoretic mobility-shift assay for HSF (heat-shock factor) activation at various temperatures in *D. virilis* strain 9 and *D. lummei* strain 200. Appearance of a high-molecular-mass HSF complex indicates HSF activation.

in essence, an independent but related phylad (the *montana* phylad) replicates the patterns of latitudinal and geographic replacement seen in the *virilis* phylad. Species within each phylad can be crossed with one another to produce partially fertile progeny; moreover, some species belonging to different phylads can also be crossed (Evgenev et al., 1982; Patterson and Stone, 1952). Second, markers present on each chromosome make possible the introgression of a single chromosome or even a portion of a chromosome bearing, for example, the *hsp70* gene cluster, whose location is known (Evgenev et al., 1978). This potential is an unexploited and exciting opportunity for evolutionary physiology.

Finally, Garbuz et al. (2002) interpreted restriction fragment length polymorphism for *hsp70* genes to suggest that the *D. virilis–D. lummei* and *D. novamexicana–D. texana* species pairs exhibit corresponding differences in *hsp70* gene family copy number. Although less likely than differences in copy number, these data are also consistent with nucleotide polymorphisms in restriction sites and a constant number of gene copies. Part II of this series of research papers will show that the *D. virilis–D. lummei* species pair indeed differ in gene copy number, with the high-latitude *D. lummei* having lost some *hsp70* genes present in the low-latitude *D. virilis.* Thus, the mRNA, protein and thermotolerance differences reported in the present manuscript have at least part of their basis in the copy number of their encoding genes.

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