Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect

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Antarctica's terrestrial environment is a challenge to which very few animals have adapted. The largest, free-living animal to inhabit the continent year-round is a flightless midge, *Belgica antarctica*. Larval midges survive the lengthy austral winter encased in ice, and when the ice melts in summer, the larvae complete their 2-yr life cycle, and the wingless adults form mating aggregations while subjected to surprisingly high substrate temperatures. Here we report a dichotomy in survival strategies exploited by this insect at different stages of its life cycle. Larvae constitutively up-regulate their heat shock proteins (small hsp, hsp70, and hsp90) and maintain a high inherent tolerance to temperature stress. High or low temperature exposure does not further up-regulate these genes nor does it further enhance thermotolerance. Such ''preemptive'' synthesis of hsps is sufficient to prevent irreversible protein aggregation in response to a variety of common environmental stresses. Conversely, adults exhibit no constitutive up-regulation of their hsps and have a lower intrinsic tolerance to high temperatures, but their hsps can be thermally activated, resulting in enhanced thermotolerance. Thus, the midge larvae, but not the adults, have adopted the unusual strategy of expressing hsps continuously, possibly to facilitate proper protein folding in a cold habitat that is more thermally stable than that of the adults but a habitat subjected frequently to freeze-thaw episodes and bouts of pH, anoxic, and osmotic stress.

Antarctica | cold tolerance | protein aggregation | temperature stress | thermotolerance

Antarctica is one of the earth's most inhospitable environments. Although an abundance of animals have adapted to life associated with the sea in this part of the world, few animals have adapted to a terrestrial existence on the continent. One exception is the midge *Belgica antarctica*, Antarctica's largest, free-living, year-round inhabitant. Prevalent in specific locations throughout the Antarctic Peninsula, *B. antarctica* feeds on dead plant material, algae, and microorganisms during its 2-yr life cycle (1, 2) and can overwinter in any of the four larval instars. The larvae are tolerant of a variety of environmental stresses and retain the ability to tolerate freezing year round (3). Wingless adults, which are not freeze-tolerant, emerge, mate in aggregations, and lay eggs for a brief period of 1–2 weeks during the summer. Summer air temperatures seldom exceed 5°C, but temperatures on the rocks, vegetation, and substrate in which the midges live may be 20°C higher (1). This study examines the high temperature responses of larvae and adults of the midge, *B. antarctica*, collected in Antarctica during January, when the insect is exposed to the temperature extremes of the austral summer.

Heat shock proteins (hsps) are well known to respond to high temperature and other environmental stresses in a wide range of organisms (4). Typically, the genes encoding these proteins are not expressed under normal conditions but are quickly turned on in response to stress and again are quickly turned off when the stress is removed. Concurrent with this up-regulation of hsps is the cessation of synthesis of most other proteins. Yet there are suggestions that some Antarctic species may respond somewhat differently. Both the Antarctic fish *Trematomus bernacchii* (5) and an Antarctic ciliate, *Euplotes focardii* (6), constitutively express *hsp70* and show no or modest up-regulation of this gene in response to thermal stress. This response is thought to be an adaptation to the cold, but constant, low temperature of the polar sea. The range of temperatures experienced in Antarctic terrestrial environments, however, is quite different from that of the ocean, and the midge faces a much broader range of temperatures in its natural habitat. In addition, larvae are frequently subjected to a variety of environmental stressors, including freezing, desiccation, osmotic stress, and anoxia (3).

Therefore, we examined the ability of *B. antarctica* to survive a range of high temperatures and examined its capacity for thermoprotection by first exposing either larvae or adults to moderately high temperatures before subjecting them to more extreme heat stress. We cloned genes encoding three hsps from the midge and investigated their expression in response to temperature stress. We report a fascinating dichotomy in which the larvae, living in a thermally buffered soil environment, express hsp transcripts continuously and lack the ability to generate further thermoprotection. Such constitutive *hsp* expression in larvae appears sufficient to prevent irreversible protein aggregation as result of a variety of environmental stresses. Alternatively, adults, which are found crawling on the surface of the substrate, up-regulate *hsps* in response to heat stress and concurrently are capable of generating thermoprotection.

Results

High-Temperature Survival. Larvae maintained in the laboratory survived at 4°C with little mortality for up to 16 days (96.6 ± 3.3% survival [mean ± SD], $n = 3$ groups of 10 each). Mortality increased at progressively higher temperatures between 15 and 30°C (Fig. 1*A*). The LT_{50} (median lethal time) was >12 days at 15°C (data not shown), whereas the calculated LT_{50} values at 20, 25, and 30°C were 100.1, 23.3, and 2.7 h, respectively.

Adults live only 1–2 weeks in the field and, in the laboratory, our field-collected samples held at 4°C survived well for 5–6 days. However, when transferred from 4 to 20°C 1–2 days after field collection, adults were considerably less heat-tolerant than

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Abbreviations: LT_{50}, median lethal time; hsp, heat shock protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ459546 (Hsp70), DQ459547 (Hsp90), DQ459548 (small Hsp), and DQ459549 (28s)].

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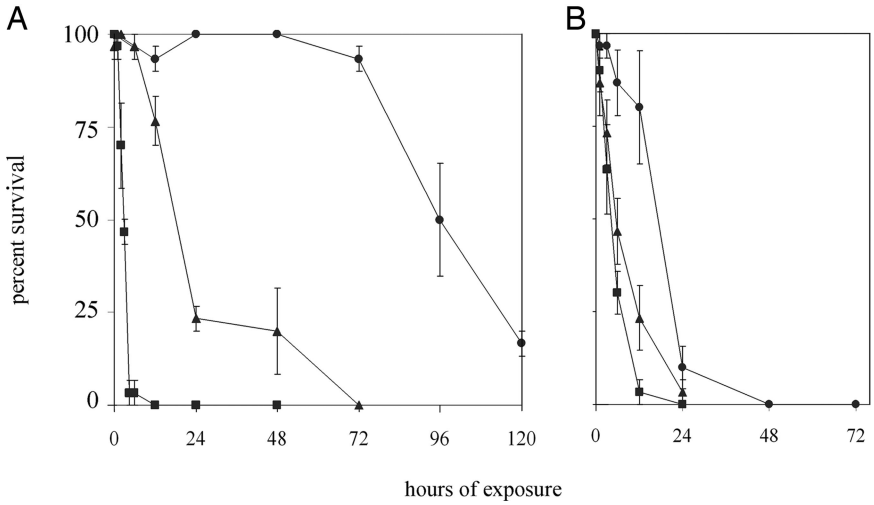

Fig. 1. Survival of larvae (*A*) and adults (*B*) of *B. antarctica* under three constant high-temperature regimes, 20°C (●), 25°C (▲), and 30°C (■). Air temperatures in the midge's habitat during the austral summer are in the range of 0–5°C, but substrate temperatures on a sunny day are occasionally as high as 25°C. Data are expressed as mean survival ± SEM; $n = 3$ groups of 10 individuals each.

larvae, surviving <1 day at 20°C (LT_{50} = 17.5 h) and for even shorter periods at higher temperatures (LT_{50} of 7.3 h at 25°C and LT_{50} of 5.4 h at 30°C) as shown in Fig. 1*B*.

Thermoprotection Generated by Prior Exposure to Less Severe High Temperatures. Survival of larvae held at 4°C and then exposed to 30°C for 3 h was 56.7 ± 3.3%. We tested the hypothesis that prior exposure to a less severe high temperature would generate thermoprotection at 30°C by first exposing the larvae for 2 h to 15, 20, or 25°C and then exposing them to 30°C for 3 h (Fig. 2*A*). A 2-h exposure to 15, 20, or 25°C, by itself, did not impair survival nor did it enhance survival of larvae place at 30°C for 3 h. In fact, the 2-h conditioning of the larvae at 15, 20, or 25°C caused thermosensitivity and reduced survival at 30°C. Likewise, a 2-h exposure to 20°C failed to protect larvae from a 1-d exposure to 25°C (Fig. 2*A*). Thus, prior exposure to a less severe thermal stress failed to elicit protection from injury at either 25 or 30°C.

Survival of adults held at 4°C and then exposed to 30°C for 6 h was 30.0 ± 5.8%. To test the capacity for generation of thermoprotection, we first exposed adults for 1 h to 20 or 25°C and then transferred them to 30°C for 6 h (Fig. 2*B*). One hour at 20 or 25°C, by itself, had little impact on survival, and 1 h at 20°C did not enhance survival at 30°C. However, 1 h at 25°C significantly increased survival at 30°C, thus indicating that adults are capable of generating thermoprotection. Our results thus indicate that the adults, but not the larvae, are capable of generating thermoprotection.

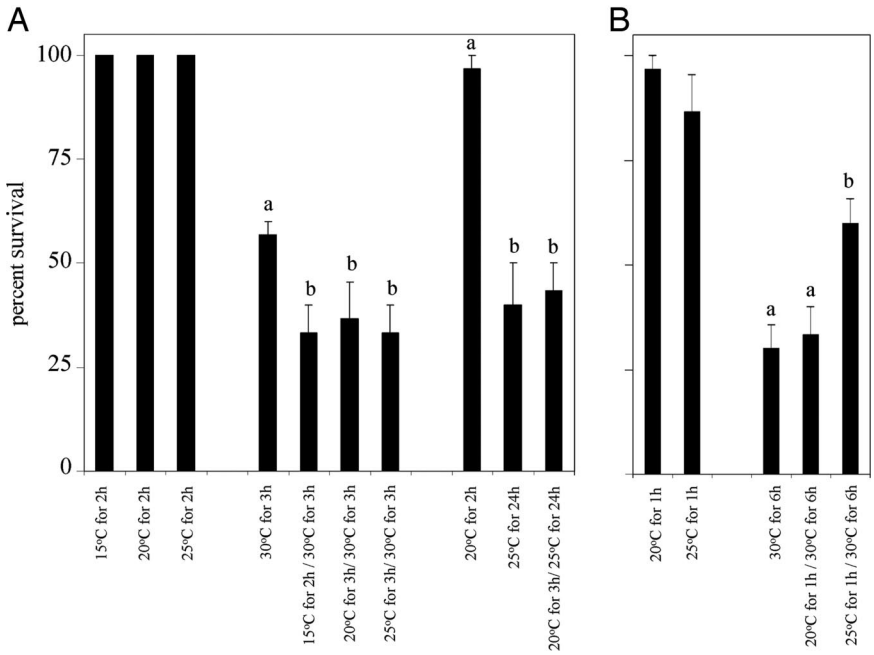

Fig. 2. Evaluation of the capacity to generate thermoprotection in larvae (*A*) and adults (*B*) of *B. antarctica* by first exposing the midges to a mildly high temperature before a higher temperature. Larvae are unable to generate thermoprotection, whereas the adults have this capacity. Survival is expressed as the mean ± SEM in three groups of 10 individuals each. Different letters indicate significant differences within each treatment series (one-way ANOVA with Tukey's honestly significant difference tests).

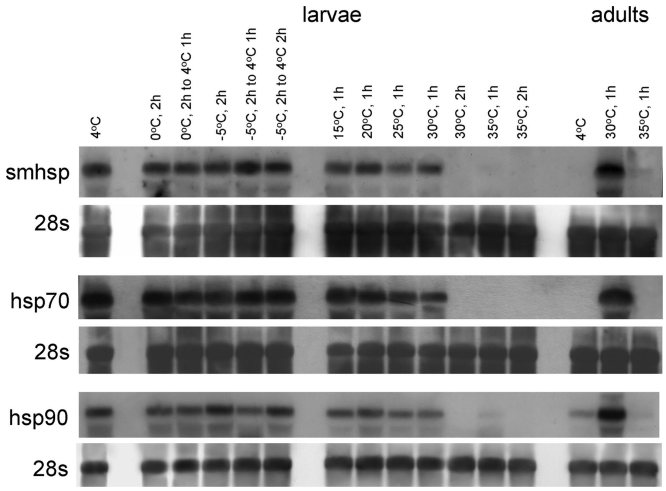

Fig. 3. Northern blot hybridization of *hsp* expression in larval and adult *B. antarctica* at ambient temperature (4°C) and in response to suboptimal and superoptimal temperatures. The larvae constituitively express their *hsps* (as documented at 4°C, a temperature typical of summer air temperatures at Palmer Station, Antarctica), but the adults do so only in response to temperature stress. For low temperature exposure, expression was assessed both immediately after the stress was incurred and after a recovery period at 4°C. Expression after heat shock was assessed immediately after the stress. As a control for equal loading, blots were stripped and rehybridized with a 28s RNA probe (GenBank accession no. DQ459549).

hsps from *B. antarctica*. Using our universal *hsp70* primers in PCR resulted in a single 231-bp product with 84% identity to *hsp70* from the mosquitoes *Anopheles gambiae* and *Culex pipiens* (GenBank accessions nos. AY137766 and AY974355, respectively). The identity to *hsp70* from the chironomid *Chironomus yoshimatsui* was a bit lower at 77%. Although this lower identity with another midge may be explained by the fact that there are multiple members of the hsp70 family, the difference was not evident at the amino acid level, where we observed 98% similarity and 100% positive identity between *B. antarctica* and *C. yoshimatsui* and 97% similarity and 100% positive identity between *B. antarctica* and the two mosquito species. This partial coding sequence of *B. antarctica hsp70* was deposited in GenBank (accession no. DQ459546).

Our consensus primers for *hsp90* also resulted in a single band of expected size from RT-PCR. The 710-bp product exhibits 78% identity to *Drosophila melanogaster*, 77% to *Anopheles albimanus*, and 75% to *Ceratitis capitata*. Conceptional translation resulted in an ORF with 83%, 86%, and 88% positive identities to *D. melanogaster*, *A. albimanus*, and *C. capitata*, respectively. The sequence of this *hsp90* clone, the first deposited *hsp90* for a chironomid, was deposited in GenBank (accession no. DQ459547).

Subtractive hybridization resulted in a 762-bp clone belonging to the small hsp family. Conceptional translation revealed a 169-aa complete coding region containing a characteristic α-crystallin domain flanked by more variable N terminus and C terminus regions. Although the similarity to described small hsps was low for the entire coding region (37% for *D. melanogaster hsp23* and 39% for *D. melanogaster hsp27*), it was somewhat higher for the 92-aa α-crystallin domain, with 41% similarity and 66% positive identity when compared with *hsp23* and with 41% similarity and 61% positive identity when compared with *hsp27*. The sequence of this small hsp is the first for a chironomid and for a terrestrial Antarctic species (GenBank accession no. DQ459548).

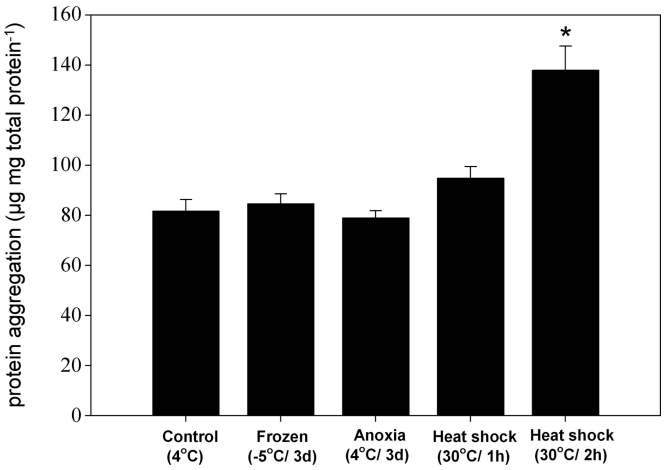

Fig. 4. Protein aggregation in larvae of *B. antarctica* maintained at 4°C or exposed to various environmental stressors. Larvae maintained low levels of protein aggregates after freezing, anoxia, or a mild heat shock. Protein aggregation increased only after a longer (2-h) heat shock. * indicates a significant difference relative to the control (one-way ANOVA with Dunnett's test). Values are the mean ± SEM of five replicates per treatment.

Expression in Response to Temperature Stress. Northern blot hybridization revealed that all three hsp transcripts (*smhsp*, *hsp70*, and *hsp90*) were expressed at high levels in larvae maintained at 4°C, whereas adults held at 4°C exhibited little or no *hsp* expression (Fig. 3). Furthermore, neither high nor low temperature exposure further increased hsp transcript abundance in the larvae. When larvae were exposed to 0 or −5°C for 2 h, none of the *hsps* showed up-regulation immediately after the stress was incurred. Often, the up-regulation of *hsps* in response to low temperature does not occur during the stress but only after a return to ambient temperature (7, 8). However, even after a recovery period at 4°C (for 1 or 2 h), *B. antarctica* larvae showed no such up-regulation. High temperature exposure of larvae also had no obvious affect on *hsp* expression. Band intensities remained constant for all three *hsps* for 1-h exposures at temperatures up to 30°C. A 2-h exposure to 30 or 35°C caused a substantial diminishment of the signal, but this was likely due to death of the insect rather than down-regulation of the *hsps.* This interpretation is supported by low-molecular-weight smearing in overdeveloped Northern blots that is present only in these lanes (data not shown) and the observed mortality of the midges.

In contrast, all three *hsps* showed little or no expression in adults maintained at 4°C, but all three genes were responsive to heat stress (Fig. 3). A 1-h exposure to 30°C resulted in high levels of expression. Little or no up-regulation was seen at 35°C, once again probably the result of the observed mortality at this temperature extreme.

Protein Aggregation in Response to Environmental Stress. Control larvae held at 4°C maintained low levels (81.7 ± 4.6 μg · mg of total protein^{-1}) of protein aggregation (Fig. 4). Exposure to a variety of environmental stresses that may result in cell injury and protein denaturation, including freezing, anoxia, and mild heat shock, did not increase protein aggregation. These results suggest that although larvae lack the typical heat shock response, constitutive hsp expression was sufficient to prevent irreversible protein aggregation that commonly occurs in response to these environmental stresses (9–11). Only after larvae were exposed to a more severe heat shock (30°C for 2 h) was protein aggregation significantly elevated (137.9 ± 9.6 μg · mg of total protein^{-1}) relative to controls.

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Discussion

Our results show that *hsp70*, *hsp90*, and a *small hsp* are continuously and strongly expressed during larval life in the Antarctic midge. This is a pattern of expression that deviates considerably from the well documented patterns of expression observed in animals from temperate and tropical regions of the world. Characteristically, the *hsps* are expressed, not at the animal's normal habitat temperature, but only as part of the organism's stress response, and in fact, the expression of these genes is thought to be incompatible with ongoing protein synthesis and the progression of development (12, 13). Thus, the continual expression of the *hsps* observed in the midge larvae presents a conundrum not easily explained by our current understanding of these proteins. The high expression we observed occurs during the brief period of the year when the midge larvae are actively feeding and growing. Presumably the midge has evolved a mechanism to maintain hsp function without disrupting normal metabolism and the growth that requires synthesis of other proteins.

Consistent with the continuous up-regulation of *hsps* is the observation that neither low nor high temperatures can further up-regulate these genes, and the mild temperature shocks that are well known to generate additional thermotolerance not only fail to boost *hsp* expression but also fail to generate thermotolerance. Thus, the *hsp* defense system already appears to be maximally expressed in the midge larvae.

This constitutive up-regulation of the *hsps* we have noted in larvae of *B. antarctica* may underscore an adaptation common to Antarctic species. *Hsp70* is also expressed constitutively in two Antarctic marine organisms, a fish (5) and a ciliate (6), and the terrestrial yeast *Candida psychrophila*, found in penguin guano, constitutively expresses both *hsp70* and *hsp90* (14). In an Antarctic grass, *Deschampsia antarctica*, *hsp70* is not expressed at its thermal optimum of 13°C, but it is expressed upon acclimation to 4°C (15), which is near the mean summer daily temperature of its habitat (16). Although far too few species have been examined to conclude that this is a common adaptation found in Antarctic organisms, it is intriguing that this type of up-regulation has been noted in several diverse species.

One other situation in which several of the *hsps* are developmentally up-regulated for months at a time is the overwintering diapause (dormancy) of temperate-zone insects (17). Transcripts of *hsp70* are up-regulated during the overwintering stages of the flesh fly *Sarcophaga crassipalpis* (7), the solitary bee *Megachile rotunda* (18), and the Colorado potato beetle *Leptinotarsa decemlineata* (8), and a *small hsp* is also up-regulated in *S. crassipalpis* (19). However, diapause is a period of developmental arrest, and the expression of *hsps* at this time may actually facilitate the suppression of development (17), a stage quite unlike the period of active growth during which *B. antarctica* expresses its *hsps*. The *hsp* expression pattern in *B. antarctica* is also unlike that of diapause because *hsp90* is up-regulated in the midge larvae, whereas this gene is either down-regulated (20) or not influenced (21, 18) by insect diapause.

In contrast to the larvae, adults of *B. antarctica* display a typical heat shock response that is thermally activated. The *hsps* are not expressed at normal habitat temperatures but can be rapidly turned on in response to either high or low temperature, and the adults can indeed generate thermoprotection against heat shock injury by first being exposed to a less severe high temperature. Why does this dichotomy exist between larvae and adults? We suspect that the difference can be attributed to differences in the thermal stability of the niches occupied by these two stages. Although the larval stage is a very long period lasting up to two years and encompassing both the austral winter and summer, the larval habitat under rocks and in the soil is a remarkably stable thermal environment. Datalogging thermocouples left in the midge's overwintering microhabitat recorded relatively mild (no less than −4°C) and stable (ranging less than 4°C) temperatures between April and September of the 2005 austral winter (data not shown). In contrast, adult midges, although they live only a couple weeks during the austral summer, experience great variability in temperature as they crawl over rock surfaces. Although air temperatures during their active period were in the 0–5°C range, temperatures of 17–19°C were common on sun-exposed surfaces, and adults were occasionally found on surfaces as warm as 22°C. Hence, our observations suggest that protection against high temperature extremes is as important to survival as protection against low temperature extremes in this Antarctic organism.

The fact that larvae, but not the adults of this species, constitutively express the *hsps* provides a clue to the rationale for the observed expression patterns. The greater thermal stability of the larva's microhabitat suggests a role for hsps in the larvae that is akin to the continuous expression of *hsp70* noted in a polar fish (5) and ciliate (6) that inhabit the cold but thermally stable polar marine environment. This pattern suggests that hsps may be uniquely used in cold, stable environments as chaperones facilitating the proper folding of proteins at low temperatures.

An alternative explanation for the constitutive expression of *hsps* is that because larvae are frequently, although unpredictably, exposed to a variety of environmental stressors such as high pH caused by penguin guano, extreme osmotic excursion, freezing, and anoxia as well as temperature (3), their survival depends on maintaining continuous expression of molecular chaperones. This maintenance prevents cell injury and irreversible protein aggregation that occurs in response to these types of stresses (9–11). This idea is supported by the data presented in Fig. 4 in which larvae that were exposed to freezing, anoxia, and mild heat shock (30°C, 1 h) exhibited low levels of protein aggregation that did not differ from control values. However, when larvae were subjected to a longer heat shock (2 h) at 30°C, protein aggregation increased significantly, suggesting that the limit of protection by constitutively expressed hsps had been exceeded. This scenario also explains the absence of a heat shock response because "preemptive" synthesis of hsps had already occurred. Furthermore, because of the unpredictability and the potential rapidity of exposure to diverse environmental stresses, the continuous production of these molecular chaperones may be energetically justified.

Materials and Methods

Insects. Fourth instar larvae (final larval instar) of *B. antarctica* were collected from field sites near penguin rookeries on Torgersen Island, near Palmer Station on the Antarctic Peninsula (lat 64°46′S, long 64°04′W) in January 2005. Soil and plant material containing the larvae were brought into the Palmer Station laboratory, and larvae were hand-picked from the substrate in ice-cold water and stored at 4°C for 2–4 days before the onset of experiments. Adults were collected from rocks and vegetation on Norsel Point and Humble Island, which are also near Palmer Station, by using an aspirator and were stored in the laboratory at 4°C for 1–3 days before being used for experiments. Daytime air temperatures at the collecting sites at the time of collection were between 0 and 5°C.

Temperature Exposure. For exposure to different temperatures, larvae or adults were placed as groups of 10 into 1.7-ml conical capped microcentrifuge tubes (VWR Scientific, West Chester, PA) with 100 μl of tap water. Each sample was run in triplicate. Temperature conditions were obtained by using refrigerated water baths (Neslab RTE-7, RTE-111, and TRE-210 units; Thermo Electron, San Jose, CA) filled with ethylene glycol. All temperature transfers were made by directly transferring the midges from one temperature to the other. When used to assess survival, the tubes

of individuals were returned to 4°C and examined for survival either 1 h (adults) or 24 h (larvae) later. For both adults and larvae, all individuals that could move were considered alive. The LT_{50} was determined from the equation calculated for the arcsine square root-transformed survival curves. Thermoprotection data were analyzed by one-way ANOVA followed by Tukey's honestly significant difference multiple-comparisons test. When used for RNA isolation, samples were pooled (25 larvae or 50 adults) before sample processing.

Gene Cloning and Sequencing. *hsp70* was cloned from *B. antarctica* by PCR, using DNA isolated from alcohol-reserved adult specimens collected by R. E. Lee during his 1981 work at Palmer Station. Preserved adults (25) were first rehydrated by using a series of incubations in diethyl pyrocarbonate-treated water, which removed ethanol from the samples that would have otherwise caused premature precipitation. They were then homogenized in DNAzol (Invitrogen, Carlsbad, CA), and DNA was extracted following standard protocol. The PCR used primers that were designed to amplify *hsp70* from a variety of insects as described (22): forward primer 5′-GATGCAGTCATCACA GTTCCAGC-3′ and reverse primer 5′-AACAGAGATC-CCTCGTCGATGGT-3′.

hsp90 was cloned upon our return from Antarctica by RT-PCR, using RNA isolated from larvae collected during the 2005 field season. Larvae (25) were maintained at 4°C before homogenization in TRIzol (Invitrogen), followed by RNA isolation using standard protocol. Amplification was accomplished by first using random primers in reverse transcription followed by PCR amplification using degenerate primers designed from the consensus sequences of several insect species (forward primer 5′-TTCGGTGTSGGTTTCTA-3′ and reverse primer 5′-TCGCAGTTGTCCATGATGAA). The PCR used Supermix (Invitrogen) following standard protocol for 35 cycles, with an annealing temperature of 50°C and an extension time of 1 min.

For use as a control in our Northern hybridizations, we also cloned a 28s ribosomal RNA fragment with RT-PCR by using the same samples as described in the cloning of *hsp90*. The PCR used primers designed from higher Diptera consensus sequences (forward primer 5′-CGAAGTTTCCCTCAGGATAG-3′ and reverse primer 5′-CCTTAAAATGGATGGCGC-3′), with an annealing temperature of 48°C and an extension time of 45 seconds for 35 cycles of amplification.

In addition to these three genes, a clone of a *small hsp* was isolated while conducting subtractive hybridization to identify genes up-regulated by stress in *B. antarctica*. Briefly, RNA from stressed individuals was subtracted against unstressed controls by using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA) by using standard protocol. This process resulted in the isolation of several unique clones (unpublished observations), one of which exhibited high identity to the small hsp family.

Northern Blotting. Each sample of total RNA was isolated from 25 larvae or 50 adults by using TRIzol reagent as described for use in Northern blot hybridization. Twenty micrograms of each sample was heat denatured and separated by electrophoresis on a 1.5% agarose, 0.41 M formaldehyde gel, transferred to a charged nylon membrane (Osmonics, Minnetonka, MN) by using a Turbo-Blotter (Schleicher and Schuell, Florham Park, NJ), and cross-linked by UV irradiation.

The aforementioned clones (small hsp, hsp70, hsp90, and 28s) were digoxigenin-labeled by using digoxigenin-high prime solution (Roche Applied Sciences, Indianapolis, IN) and were then used as probes in Northern hybridization by using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences) following standard protocol. BioMax Chemiluminescence film (Kodak, Rochester, NY) was then exposed to the blot for signal detection. Equal loading of samples was confirmed by alkaline stripping the membrane by using 0.2 M NaOH, 0.1% SDS followed by reprobing with the 28s rRNA probe. All Northerns were run in triplicate.

Protein Aggregation. Protein aggregates were isolated by differential centrifugation as described in Chen *et al.* (11). Briefly, the Triton-insoluble fraction was twice resuspended, sonicated, and pelleted at 17,000 × *g* for 30 min at 4°C. The resulting pellet was again resuspended, sonicated, and pelleted at 5,000 × *g* for 30 min at 4°C to purify the protein aggregate fraction. Then the pelleted protein aggregates were resuspended and assayed for protein content. Protein concentrations were determined by using the Bradford procedure (BioRad, Richmond, CA) using BSA as a standard.

Heat shock and freezing treatments were performed with groups of 10 larvae placed in 1.7-ml microcentrifuge tubes with 100 μl of distilled water. For anoxia treatment, groups of ≈40 larvae were placed in 15-ml vials filled with distilled water, in which anoxic conditions were created by bubbling N_2 gas into the vials for several minutes. Vials were subsequently sealed and placed at 4°C for the duration of the anoxic exposure. After all experimental treatments, larvae were held for 1 h at 4°C before measurement of protein aggregation. Five replicates of ≈40 larvae were used for each determination of protein aggregation.

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