

Fig. S2. Phylogenetically-independent contrasts (PICs) of the critical thermal minimum (CT_{\min}) regressed against hemolymph ion concentrations (A,B), hemolymph osmolality (C) and Na^+/K^+ -ATPase parameters (D-F). P -values were obtained from linear regressions of PIC data forced through the origin (intersection of dotted grey lines). Solid black lines denote a significant relationship between the PICs of CT_{\min} and hemolymph Na^+ and K^+ concentration. T_s : thermal sensitivity; IP: inflection point.

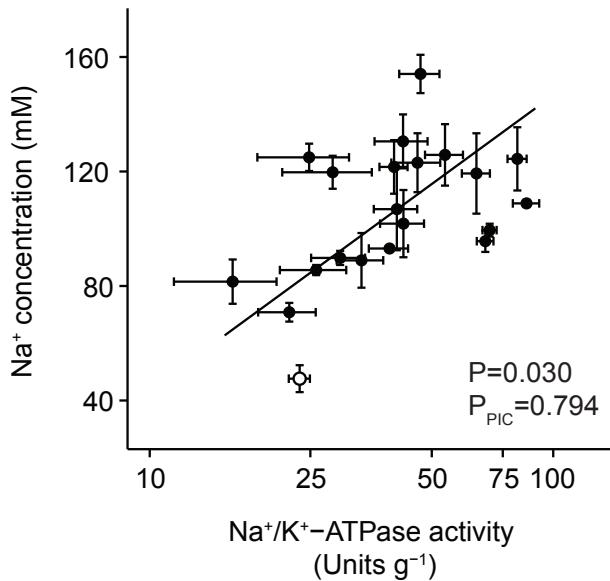


Figure S3. Mean (\pm sem) hemolymph Na^+ concentration in relation to maximal Na^+/K^+ -ATPase activity at 21.5 °C (log₁₀ scale) of 21 species of the genus *Drosophila*. Solid line denotes a significant relationship between Na^+ concentration in the hemolymph and log-transformed pump activity among species (line equation generated by model II regression). *Drosophila immigrans* (open circle) was included in this analysis, but had both low Na^+/K^+ -ATPase activity and low hemolymph $[\text{Na}^+]$. Hemolymph $[\text{Na}^+]$ was a poor predictor of cold tolerance for this species (see main text for further discussion). P : P -value of correlation of species means. P_{PIC} : P -value from a regression of phylogenetically-independent contrasts forced through the origin. $n=3-6$ (Na^+/K^+ -ATPase activity) and 3-5 (Na^+) biological replicates per species.

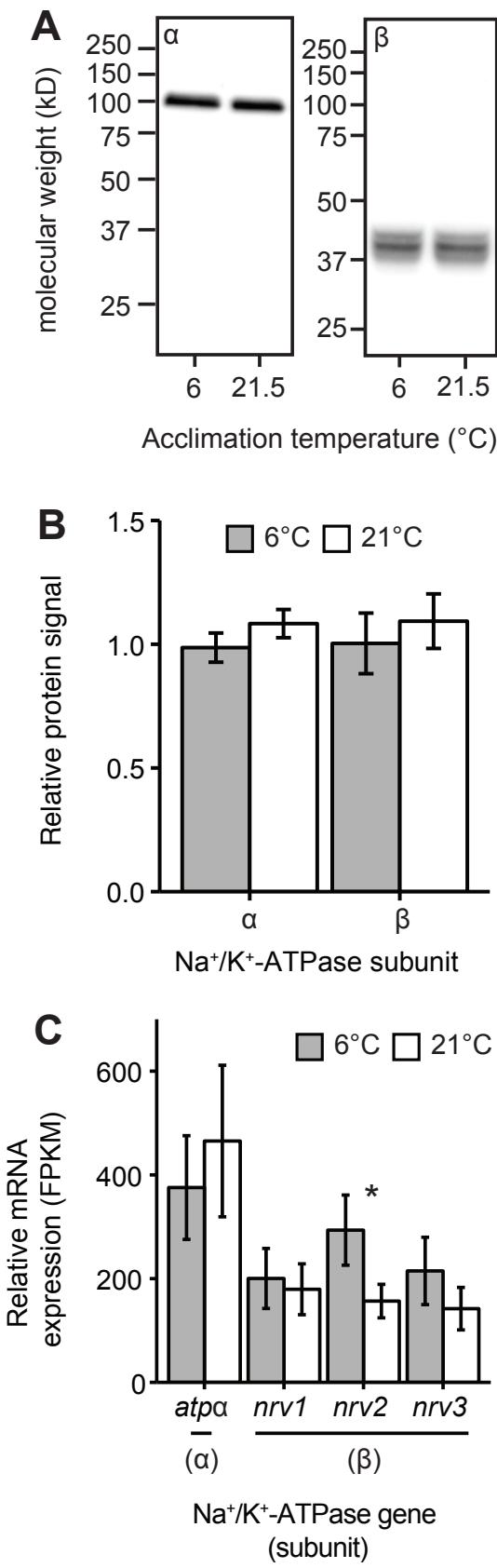


Fig. S4. Na^+/K^+ -ATPase protein abundance and mRNA expression in warm- and cold-acclimated *Drosophila melanogaster*. (A) Representative immunoblots of the α (~110 kD) and β (37-42 kD) subunits of Na^+/K^+ -ATPase of *D. melanogaster* acclimated to 6 °C and 21.5 °C. (B) Mean \pm sem relative protein abundance as determined from immunoblot lane intensity. Samples (15 μg soluble protein) and signal expressed as relative abundance corrected to total protein staining. n=7 (α) and 4-5 (β) per acclimation group. Warm- and cold- acclimated flies did not differ significantly in the abundance of either Na^+/K^+ -ATPase subunit. (C) Na^+/K^+ -ATPase subunit transcript expression, expressed as fragments per kilobase of transcript per million mapped reads (FPKM \pm 95 % CI). Asterisk denotes a significant difference in relative expression of *nrv2* between acclimation groups. n=5 biological replicates of 25 flies per acclimation group.

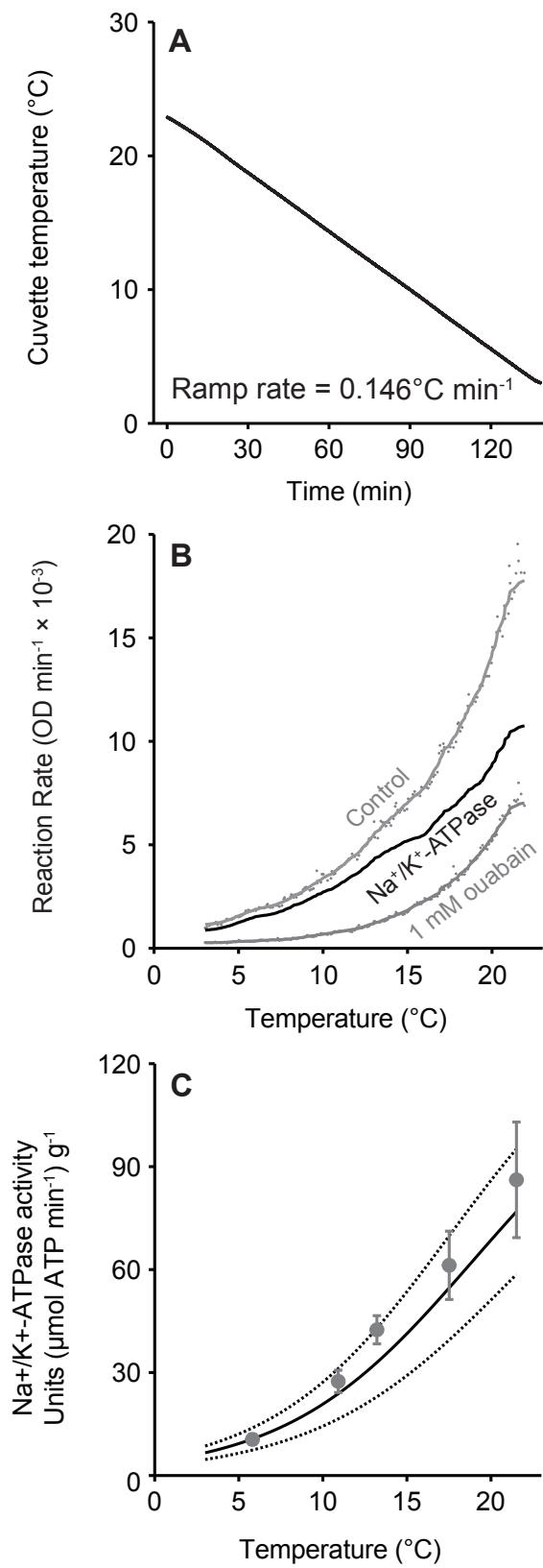


Fig. S5. $\text{Na}^{+}/\text{K}^{+}\text{-ATPase}$ activity recorded using a temperature ramp and comparison to activity measured at static temperatures. (A) Temperature of a dummy sample recorded by a type T-thermocouple held inside a cuvette during a $\text{Na}^{+}/\text{K}^{+}\text{-ATPase}$ activity determination. Temperature ramps accomplished within the spectrophotometer were highly linear, allowing for accurate calculation of instantaneous reactions rates with regard to temperature. (B) Reaction rates measured using a dynamic ramping technique (grey points) were smoothed using a sliding window method (grey lines) before rates of ouabain-inhibited samples were subtracted from the same samples without ouabain to yield activity of $\text{Na}^{+}/\text{K}^{+}\text{-ATPase}$ (black line). (C) Comparison of $\text{Na}^{+}/\text{K}^{+}\text{-ATPase}$ activities measured using the ramping technique (black lines) to activity measured at static temperatures (grey points). Dynamically ramped samples are expressed as a mean rate \pm 95 % CI (dotted lines) determined from logistic fits of $n=6$ samples. Static temperature data is expressed as mean \pm sem of $n=4$ samples at each temperature.

Table S1. Details of *Drosophila* species stock origins. The majority of species were obtained from the UC San Diego and Ehime University *Drosophila* stock centers. Species names in bold have had their genome sequenced as part of the *Drosophila* 12 genomes project. Stars denote the strain is the same as that which was used for genome sequencing.

| Species | Source ¹ | Stock No. | Origin Location | Collection Date |
|--------------------------------|---------------------|---------------|-----------------------------------------|-----------------|
| <i>D. algonquin</i> | UCSD | 14012-0161.03 | Rocky Point, New York, USA | 2004 |
| <i>D. ananassae</i> | EU | E-11002 | Kagoshima, Japan | 2001 |
| <i>D. arawakana</i> | UCSD | 15182-2261.03 | Monkey Hill, St. Kitts, Caribbean | 2005 |
| <i>D. auraria</i> | EU | E-11220 | Hokkaido, Japan | 2004 |
| <i>D. borealis</i> | UCSD | 15010-0961.05 | Lytton, Quebec, Canada | 1949 |
| <i>D. erecta*</i> | UCSD | 14021-0224.01 | Unknown (Afrotropical species) | Prior to 1977 |
| <i>D. funebris</i> | EU | E-13501 | Hokkaido, Japan | 1987 |
| <i>D. hydei</i> | Field | NA | London, Ontario, Canada | 2007 |
| <i>D. immigrans</i> | UCSD | 15111-1731.03 | Patan, Nepal | Prior to 1977 |
| <i>D. kanekoi</i> | EU | E-13901 | Hokkaido, Japan | 1987 |
| <i>D. mauritiana</i> | AM | NA | Mauritius | 1981 |
| <i>D. melanogaster</i> | Field | NA | London, Ontario, Canada | 2007 |
| <i>D. mercatorum</i> | UCSD | 15082-1521.25 | Argoim, Bahia, Brazil | 1976 |
| <i>D. mojavensis*</i> | UCSD | 15081-1352.22 | Santa Catalina, California, USA | 2002 |
| <i>D. nepalensis</i> | RP | NA | Shimla, India | 2004 |
| <i>D. obscura</i> | UCSD | 14011-0151.00 | Heidelberg, Germany | 2005 |
| <i>D. persimilis*</i> | UCSD | 14011-0111.49 | St. Helena, California, USA | 1997 |
| <i>D. pseudoobscura</i> | UCSD | 14011-0121.94 | Mesa Verde, Colorado, USA | 1996 |
| <i>D. sechellia*</i> | UCSD | 14021-0248.25 | Cousin Island, Seychelles | 1980 |
| <i>D. takahashii</i> | EU | E-12201 | Okinawa, Japan | 2002 |
| <i>D. triauraria</i> | UCSD | 14028-0691.01 | Meguro, Tokyo, Japan | Unknown |
| <i>D. virilis</i> | EU | E-15601 | Hokkaido, Japan | 1985 |
| <i>D. willistoni*</i> | UCSD | 14030-0811.24 | Guadeloupe island, Caribbean | Unknown |
| <i>D. yakuba*</i> | UCSD | 14021-0261.01 | Between Liberia and Ivory Coast, Africa | 1983 |

1. UCSD: University of California San Diego Stock Center; EU: Ehime University Stock Center; Field: Field collected; AM: Dr. Amanda Mohering, Western University; RP: Dr. Ravi Parkash, Maharshi Dayanand University, India.

Table S2. Summary of physiological variables measured in *Drosophila* species (mean \pm sem). CT_{min} is the temperature at which 80 % of flies fell from the column based on accelerated failure time models (section 6.2.8). Maximal rate of Na⁺/K⁺-ATPase (V_{max}) at 21.5 °C is expressed as Units ($\mu\text{mol ATP min}^{-1}$) per g of protein. nm: not measured. Ts: thermal sensitivity of Na⁺/K⁺-ATPase. IP: temperature of inflection point of Na⁺/K⁺-ATPase activity (see Fig. 4).

| Species | CT _{min} (°C) | Hemolymph | | | Na ⁺ /K ⁺ -ATPase | | |
|-------------------------|------------------------|-------------------------|------------------------|-------------------|-----------------------------------------|------------|-------------|
| | | [Na ⁺] (mM) | [K ⁺] (mM) | Osmolality (mOsm) | V _{max} at 21.5°C | IP (°C) | Ts |
| <i>D. algonquin</i> | -1.79 ± 0.08 | nm | nm | nm | 50.3 ± 7.1 | 23.9 ± 4.0 | 6.20 ± 0.53 |
| <i>D. ananassae</i> | 7.36 ± 0.04 | 99.5 ± 2.2 | 35.5 ± 4.3 | 450 ± 14 | 62.8 ± 7.5 | 18.1 ± 2.2 | 4.49 ± 0.57 |
| <i>D. arawakana</i> | 7.30 ± 0.08 | 111.2 ± 16.9 | 27.2 ± 2.2 | 344 ± 11 | nm | nm | nm |
| <i>D. auraria</i> | 2.45 ± 0.07 | 119.7 ± 5.8 | 16.8 ± 1.3 | 589 ± 14 | 28.4 ± 5.9 | 14.4 ± 0.9 | 4.06 ± 0.29 |
| <i>D. borealis</i> | -2.90 ± 0.09 | 85.6 ± 1.8 | 17.7 ± 2.6 | 459 ± 28 | 25.9 ± 5.2 | 16.5 ± 2.5 | 4.04 ± 0.67 |
| <i>D. erecta</i> | 7.17 ± 0.07 | 119.3 ± 14.0 | 23.2 ± 1.9 | 582 ± 43 | 64.5 ± 9.8 | 18.8 ± 1.5 | 4.53 ± 0.44 |
| <i>D. funebris</i> | 1.36 ± 0.09 | 81.5 ± 7.7 | 17.8 ± 2.6 | 387 ± 29 | 14.4 ± 1.7 | 18.2 ± 4.9 | 4.82 ± 1.11 |
| <i>D. hydei</i> | 1.38 ± 0.07 | 89.0 ± 9.6 | 20.1 ± 1.5 | 436 ± 31 | 33.5 ± 6.4 | 18.4 ± 2.0 | 4.35 ± 0.85 |
| <i>D. immigrans</i> | 8.17 ± 0.08 | 47.6 ± 4.7 | 33.7 ± 5.4 | 645 ± 34 | 23.5 ± 3.3 | 18.9 ± 2.0 | 4.55 ± 0.27 |
| <i>D. kanekoi</i> | -1.40 ± 0.08 | nm | nm | nm | 17.4 ± 2.6 | 19.3 ± 3.2 | 4.40 ± 0.94 |
| <i>D. mauritiana</i> | 2.55 ± 0.05 | 106.8 ± 14.4 | 21.2 ± 0.8 | 432 ± 31 | 50.4 ± 10.1 | 18.3 ± 0.8 | 4.86 ± 0.40 |
| <i>D. melanogaster</i> | 1.81 ± 0.07 | 125.8 ± 10.7 | 21.6 ± 2.7 | 331 ± 27 | 68.9 ± 12.6 | 18.5 ± 1.6 | 4.05 ± 0.76 |
| <i>D. mercatorum</i> | 4.57 ± 0.06 | 89.8 ± 2.5 | 19.8 ± 1.6 | 343 ± 13 | 26.3 ± 6.4 | 24.3 ± 4.8 | 6.10 ± 0.90 |
| <i>D. mojavensis</i> | 2.75 ± 0.08 | 124.9 ± 4.8 | 19.7 ± 1.6 | 423 ± 44 | 24.8 ± 1.6 | 23.4 ± 4.5 | 5.98 ± 0.37 |
| <i>D. nepalensis</i> | 3.82 ± 0.09 | 95.6 ± 3.4 | 29.6 ± 2.6 | 388 ± 36 | 57.4 ± 20.2 | 22.8 ± 3.1 | 4.81 ± 0.96 |
| <i>D. obscura</i> | -1.69 ± 0.09 | 93.1 ± 0.1 | 21.2 ± 2.6 | 380 ± 15 | 53.9 ± 8.6 | 18.6 ± 1.9 | 4.76 ± 0.40 |
| <i>D. persimilis</i> | 0.16 ± 0.10 | 123.1 ± 10.3 | 19.3 ± 1.0 | 462 ± 7 | 46.1 ± 7.1 | 18.8 ± 2.7 | 4.95 ± 0.63 |
| <i>D. pseudoobscura</i> | -0.67 ± 0.10 | 101.8 ± 11.7 | 19.2 ± 2.5 | 542 ± 40 | 48.6 ± 8.7 | 18.4 ± 1.1 | 4.94 ± 0.20 |
| <i>D. sechellia</i> | 7.00 ± 0.07 | 154.1 ± 6.7 | 28.7 ± 3.2 | 526 ± 8 | 45.1 ± 2.0 | 26.0 ± 2.4 | 6.51 ± 0.60 |
| <i>D. takahashii</i> | 6.50 ± 0.04 | 124.4 ± 11.0 | 28.0 ± 6.4 | 352 ± 34 | 63.2 ± 12.8 | 21.7 ± 1.9 | 5.55 ± 0.45 |
| <i>D. triauraria</i> | 1.95 ± 0.10 | 130.5 ± 9.4 | 20.5 ± 2.7 | 489 ± 59 | 41.8 ± 2.3 | 24.0 ± 4.8 | 5.75 ± 0.66 |
| <i>D. virilis</i> | -0.49 ± 0.07 | 70.8 ± 3.3 | 19.6 ± 2.6 | 327 ± 45 | 25.8 ± 5.2 | 19.3 ± 1.5 | 4.72 ± 0.23 |
| <i>D. willistoni</i> | 7.88 ± 0.06 | 121.6 ± 9.4 | 37.6 ± 2.8 | 468 ± 16 | 37.0 ± 5.3 | 16.9 ± 1.4 | 4.74 ± 0.38 |
| <i>D. yakuba</i> | 6.02 ± 0.05 | 108.9 ± 1.2 | 16.9 ± 1.5 | 297 ± 2 | 75.4 ± 13.1 | 23.0 ± 3.9 | 5.47 ± 0.80 |