# Supporting Information

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## SI Materials and Methods

***Drosophila* Stocks and Generation of Transformants.** Wild-type *Canton-S* (CS) and cantonized *white*$^{1118}$, *UAS-gfp*, *UAS-capa RNAi* (TRIP line), original P insertion into *vha55* and *capa-Gal4*$^{2}$ flies were obtained from Bloomington Stock Center. *Va-Gal4* was a kind gift of Stefan Thor (Linköping University, Linköping, Sweden). The *capaR-GAL4* and the *UAS-capaR RNAi* lines were previously generated in house (1). The P-element insertional *nha1* mutant and a control PBac strain were obtained from The Exelixis Collection at the Harvard Medical School. *Drosophila montana* was from lines collected in North Finland in 2009 (2). *Drosophila mojavensis* was a line (A976) derived from a stock collected in Baja California Sur, Mexico by William Etges in 1996. To generate the *capa-Gal4*$^{1}$ driver, the putative promoter sequence of the *capa* gene was amplified from *D. melanogaster* wild-type genomic DNA as a template with the primer pairs described in Table S4. The resulting amplicon (corresponding to bases −1 to −268 from the transcriptional start site of the *capa* coding region) was digested with KpnI and subcloned into pinGal4 vector (1), which was previously digested with KpnI and treated with calf-intestinal alkaline phosphatase. Transgenic line was generated using standard methods for P-element–mediated germline transformation (BestGene Inc.).

**Quantitative RT-PCR.** Quantitative RT-PCR (qRT-PCR) amplifications were performed using the *capa* probe (Dm02151769_g1) with TaqMan Gene Expression Assays products in an ABI StepOnePlus Detection System (Applied Biosystems). For validation in *Drosophila* species, *Aedes*, and *Anopheles capa* mRNA levels, qRT-PCR was performed using Opticon DNA engine 4 (Bio-Rad Technologies) using Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent) with the primer pairs listed in Table S4. Data were normalized against the *rp49* standard and expressed as fold change compared with controls ± SEM ($n$ = 3).

**Malpighian Tubule Fluid Transport Assays.** Fluid droplets were collected every 10 min, and secreted fluid volumes were calculated from live, intact tubules dissected from 7-d-old male *D. melanogaster* wild-type flies or *capa* transgenic flies. Basal fluid secretion rates were monitored for 30 min; then $10^{-7}$ M *Drm*-capa-1 or *Manse*-CAP2b analogs were added, and the secretion rate was recorded for a further 30 min.

**Immunofluorescence.** Larval and adult nervous systems were dissected in Schneider's medium (Invitrogen) and fixed with 4% (wt/vol) paraformaldehyde for 30 min at room temperature. Mouse anti-GFP primary antibody (1:500; Zymed), mouse anti-nc82 (1:100; Developmental Studies Hybridoma Bank), and the rhodamine-labeled purified rabbit anti-capa precursor peptide (3) (1:300) were used. Incubations in the primary antibodies were performed overnight. Texas red- or FITC-conjugated affinity-purified goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch) were used in a dilution of 1:1,000 for visualization of the primary antiserum. All samples were mounted in Vectashield (Vector Laboratories). Confocal images were taken with a Zeiss LSM META 510 microscope and processed with LSM 510 image examiner and Adobe Photoshop CS 5.1.

**Gravimetric Estimates of Body Water.** To measure wet body weight, individual normally fed 7-d-old male *D. melanogaster* flies were anesthetized on ice and weighed on an AND GR-202 precision balance (analytical weighing to within 0.0001 g). Another group of flies of each genotype were weighed after 24 h desiccation with no food or water. For dry body weight, flies were killed by freezing at −80 °C for 20 min and subsequently were dried at 60 °C for 24 h. Dry flies were weighed after reaching room temperature. The weight of total body water was calculated by subtracting dry weight from wet weight. Water loss over 24 h was calculated for each genotype by subtracting water content at 24 h from that at 0 h. Experiments were run in triplicate with at least 30 flies of each genotype ($n$ = 90–110 flies for the three genotypes).

**Assays of Gene Expression and/or Survival for Different Stressors.**

***Immune.*** *D. melanogaster* wild-type (*Canton-S*) 7-d-old male flies were anesthetized briefly with $CO_2$ and then were injected with Gram-negative *E. coli* bacteria or Gram-positive *B. subtilis* bacteria (immune challenge) according to the protocol previously described (4).

***Oxidative, osmotic, and ionic stress, starvation, and desiccation.*** *D. melanogaster* wild-type 7-d-old male flies were anesthetized briefly with $CO_2$ and then were placed in groups of 30–40 in 30-mL cotton-capped glass vials containing 5 mL 1% aqueous agarose/1% sucrose medium (control) or were fed for 24 h with either 1% $H_2O_2$ (5), 0.8 M sorbitol (6), or 0.6 M NaCl (7). For starvation, 7-d-old male flies were anesthetized briefly with $CO_2$ and then were transferred in vials containing 1% aqueous agarose only. All vials were placed in an incubator at 22 °C, 55% humidity with a 12:12 light:dark photoperiod. Vials were checked for dead flies every 6–12 h until no living flies remained. Mortality was determined by the inability of the flies to resume an upright position after the vial was shaken. For desiccation experiments the same protocol was followed, except that the vials were empty (no food or water), and the open end of the tube was sealed with parafilm (Bemis, NA). Vials were checked hourly until no living flies remained. All experiments were run in triplicate with at least 30 flies in each run of specified genotype. For mosquito desiccation, 4- to 6-d-old male *Aedes aegypti* and *Anopheles gambiae* mosquitoes were fed 5% sucrose medium (control) or were desiccated for 12 h (corresponding to the median survival time of adult mosquitoes). Mosquitoes were placed into a 50-mL tube (BD Biosciences) in groups of 10 with a cotton ball on which the mosquitoes could rest. Tubes were capped, sealed with parafilm, and incubated at 22 °C.

***Heat shock.*** *D. melanogaster* wild-type 7-d-old male flies were either untreated or exposed to heat stress (36 °C for 1 h; no mortality was observed) (8) and then were transferred immediately to microcentrifuge tubes for extraction of RNA and qRT-PCR analysis.

***Cold shock and RCH experiments.*** *D. melanogaster* adults were tested for differences in their intrinsic cold shock tolerance and their ability to undergo RCH. RCH is a physiological response by which insects significantly enhance cold shock tolerance following only brief exposure to nonlethal low temperature. To assess cold shock tolerance, flies were exposed directly to −6 °C for 1 h in a programmable cold bath. After exposure, flies were placed in vials with a small amount of food and allowed to recover for 24 h. Flies that could stand on all six legs and walk forward, either spontaneously or with gentle prodding, were considered to be alive. To test for RCH, flies first were exposed to +4 °C for 2 h and then were transferred immediately to −6 °C for 1 h. For each treatment condition, we exposed four or five replicate vials, for a total of 89–123 flies per treatment group.

***Chill coma recovery.*** "Chill coma" refers to the reversible loss of neuromuscular function at low temperature caused by the inability to maintain ion homeostasis and cellular water balance at low temperature. For these experiments, *D. melanogaster* adults were

transferred to empty vials and exposed to 0 °C for varying amounts of time by suspending the vials in a slurry of ice water. After cold exposure, flies were transferred immediately to filter paper at 22 °C and monitored for recovery time. A fly was considered to have recovered when it had righted itself fully and was standing on all six legs. Recovery times were recorded for each individual fly. $n$ = 19–22 flies per treatment for initial experiment with varied exposure times, and $n$ = 44–50 flies per treatment for subsequent CCR experiments.

**Peptide Analog Nanoinjections.** *Manse*-CAP2b [$Ala^3$], 883.9 [$M + H^+$] and *Manse*-CAP2b [$Ala^7$], 890.6 [$M + H^+$] peptides were as described (9). Peptides were resuspended in saline injection buffer [pH 6.7, (in mmol $l^{-1}$) NaCl 117.5, KCl 20, $CaCl_2$ 2, $MgCl_2$ 8.5, $NaHCO_3$ 10.2, $NaH_2PO_4$ 4.3, Hepes 15, glucose 20]. Seven-day-old *capa* > *capa RNAi* flies were preexposed to 0 °C for 4 h or were not preexposed and were injected with ∼69 nL of $10^{-6}$ M peptide solution using a Nanoject II apparatus (Drummond Scientific) under a Zeiss dissecting microscope. Flies were left to recover postinjection for 30 min before desiccation. For each treatment condition, we exposed three or four replicate vials containing 30 flies.

**Data Analysis.** For *capa* mRNA level quantification or fluid secretion analysis, a two-tailed Student's $t$ test (for two independent groups (nonstressed vs. stressed or basal vs. stimulated) was used. For water retention assays, one-way ANOVA (for three independent groups: *capa*/+ vs. *capa RNAi*/+ vs. *capa* > *capa RNAi*) was used to compare the water loss levels in desiccated flies. For wet-, desiccated-, and dry-weight measurements, two-way ANOVA was used to compare weights for each genotype and condition. For both ANOVAs, $P$ values were adjusted with the Sidak multiple comparisons test. For survival curves obtained in starvation and desiccation assays, significance was assessed by the log-rank (Mantel–Cox) test. For cold shock experiments, we fit a generalized linear mixed model (GLMM) with proportion surviving as the response variable and line, sex, and temperature treatment (cold shock or RCH) as the factors, with vial nested within line as a random effect. The model was fit assuming a binomial distribution and a logit link function, using the glmer function in the lme4 package in R. After determining the general effects of each variable, we fit a saturated model and conducted pairwise comparisons using linear contrasts with the glht function from the multcomp package in R. $P$ values from the linear contrasts were adjusted with the Benjamini–Hochberg false-discovery rate method. For the chill coma experiment with varied exposure times, we fit a Cox proportional hazards model with CCR as a function of exposure time, line, and their interaction. Risk ratio tests were used to assess differences in CCR between lines across the range of exposure times. For subsequent CCR experiments, recovery curves for each were compared using the Mantel–Cox log-rank test. Log-rank tests were conducted for each pairwise comparison. All statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc.).


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2. Veltsos P, Wicker-Thomas C, Butlin RK, Hoikkala A, Ritchie MG (2012) Sexual selection on song and cuticular hydrocarbons in two distinct populations of Drosophila montana. *Ecology and Evolution* 2(1):80–94.
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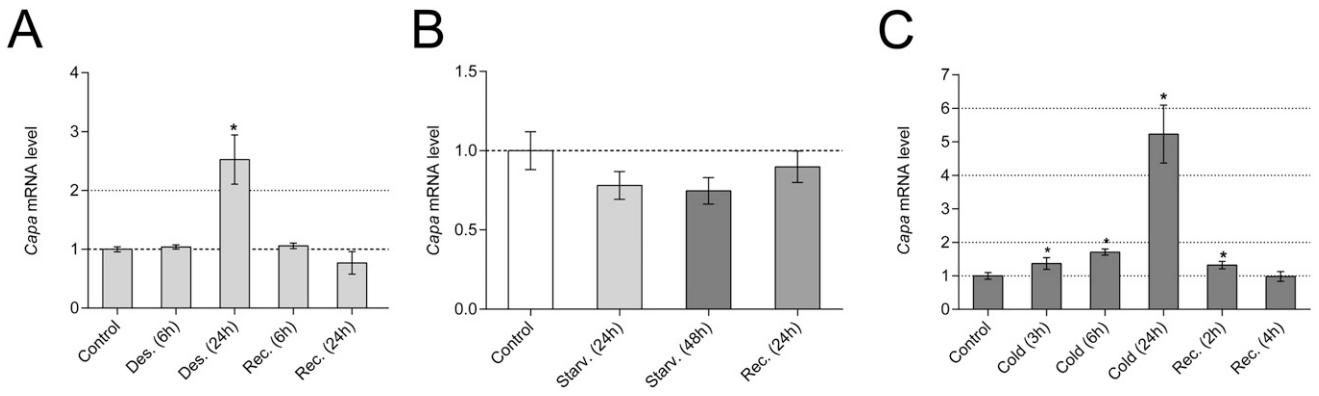


**Fig. S1.** *Capa* mRNA expression during desiccation, starvation, cold, and recovery time. (*A*) *D. melanogaster* flies were subjected to 6 and 24 h desiccation (Des.) and were left to recover (Rec.) in food vials for 6 and 24 h. (*B*) No significant differences in *capa* mRNA expression were observed when wild-type flies were starved for either 24 or 48 h and were left to recover in normal food for 24 h. (*C*) Wild-type flies were cold stressed at 0 °C to induce chill coma for 3, 6, and 24 h and were allowed to recover at 22 °C for 2 and 4 h. Expression levels of *capa* were normalized against the housekeeping reference *rp49*, and values are expressed as fold change relative to control ± SEM ($n$ = 3). Asterisks indicate a significant difference from control ($P < 0.05$ as determined by Student's $t$ test).

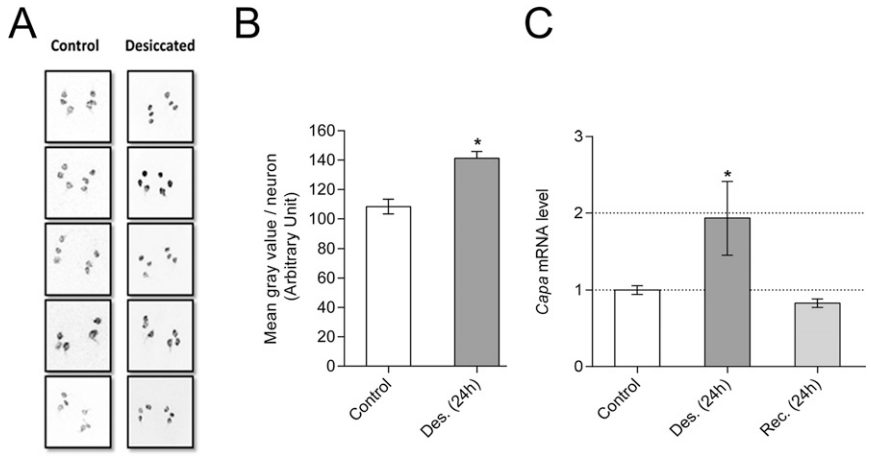


**Fig. S2.** Capa precursor levels and transcripts are increased in response to desiccation stress. (*A* and *B*) Intensity of capa precursor immunofluorescence in three pairs of Va neurons of desiccated larvae is significantly higher than in controls as measured by the gray value intensity/neuron on merged Z-stack acquired using a Zeiss LSM META 510 microscope and determined with Image J. (*C*) Larvae subjected to 24 h desiccation have significantly higher *capa* mRNA expression than controls. The levels returned to basal when larvae were given access to food and water. Expression levels of *capa* are expressed as fold change relative to control ± SEM ($n = 3$). In *B* and *C*, asterisks indicate a significant difference from control ($P < 0.05$ as determined by Student's *t* test).

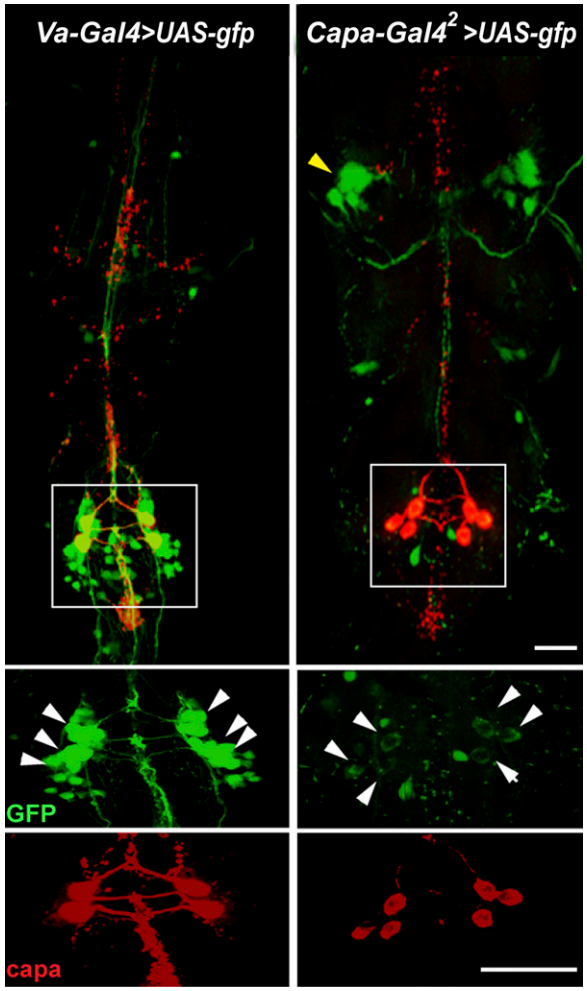


**Fig. S3.** *Va-Gal4* and *capa-Gal4*$^2$ drive gene expression in capa-expressing neurons. Immunocytochemical localization of the capa neurons, labeled using an antibody against capa precursor (red) in adult thoracoabdominal ganglion of *Va-Gal4* > *UAS-gfp* (*Left*) and *capa*$^2$*-Gal4* > *UAS-gfp* (*Right*) progeny. (*Insets*) Higher magnification of the three pairs of abdominal neuroendocrine cells colocalize (yellow, merge). (Scale bar, 100 μm.)

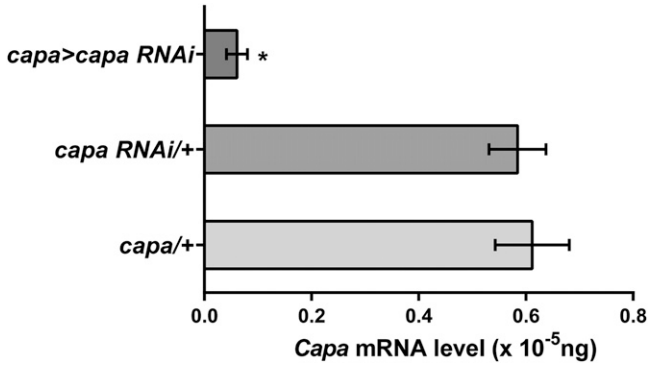


**Fig. S4.** Validation of *capa* RNAi knockdown. *Capa* mRNA in adult CNS is reduced significantly in *capa* > *capa RNAi* compared with both *capa RNAi*/+ and *capa*/+ parental lines (*$P < 0.05$ as determined by Student's *t* test). The observed difference in sample quantity corresponds to an ∼90% decrease in *capa* mRNA levels. Data are expressed as $10^{-5}$ ng of *capa* mRNA ± SEM ($n = 3$).

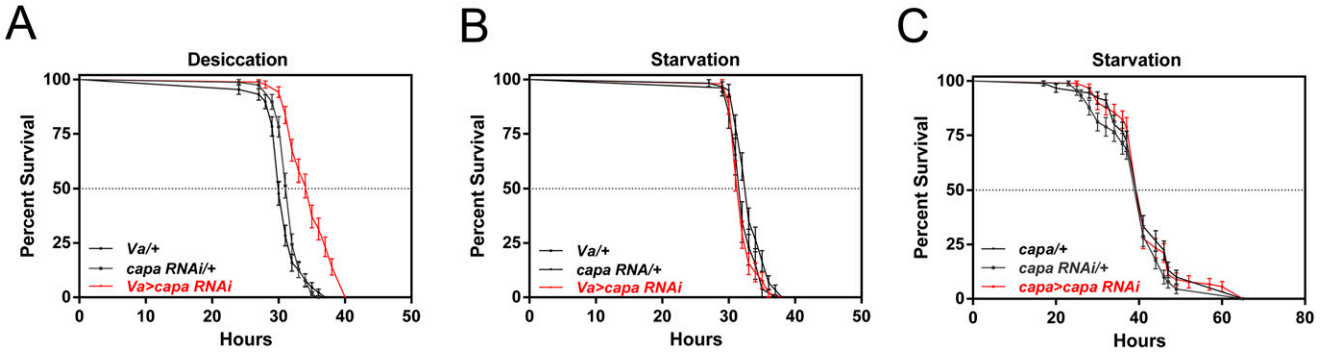


**Fig. S5.** Knockdown of *capa* expression enhances organismal survival in desiccation but not in starvation. Reduced capa levels in the abdominal ventral neurons (*Va* > *capa RNAi*, red trace) alter survival of desiccated flies (*A*), but not of starved flies *Va* > *capa RNAi* (*B*) or *capa* > *capa RNAi* (*C*). In *A*, stress tolerance was significantly higher after knockdown of *capa* than in controls ($P < 0.001$ against both controls; log-rank test; $n = 100–130$ for the different genotypes).

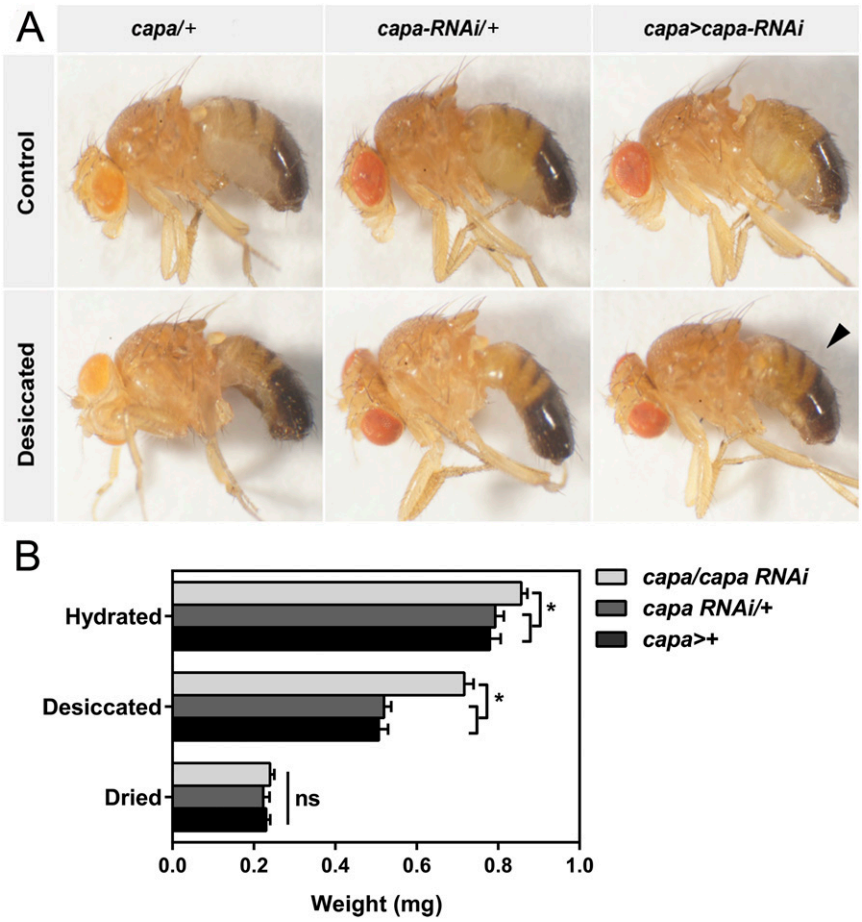


**Fig. S6.** Distended abdomen phenotype and weight measurements in *capa*-knockdown male flies exposed to desiccation. (*A*) Phenotypes of *D. melanogaster* either normally fed (control) or desiccated for 24 h. After desiccation, flies with reduced capa levels (*capa* > *capa RNAi*) exhibit a larger abdominal volume (arrowhead) than parental controls, likely indicating higher volumes of hemolymph. (*B*) Wet weights of fully hydrated flies and weights after 24 h desiccation were significantly higher in *capa*-knockdown flies than in parental controls. Data are expressed as milligrams ± SEM (two-way ANOVA, with Sidak multiple comparisons test, $*P < 0.05$; $n = 90–110$ flies for each genotype). ns, not significant.

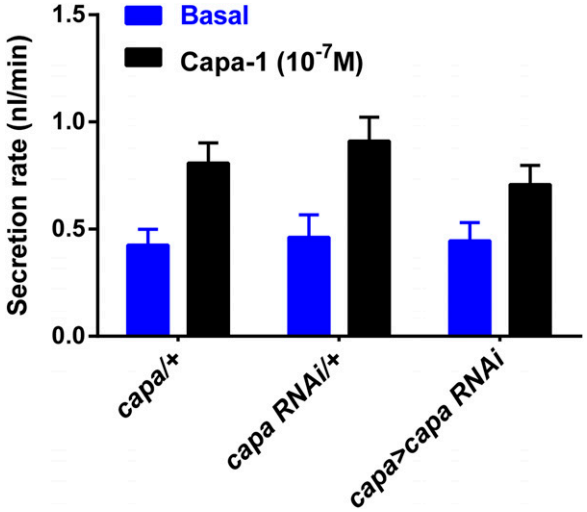


**Fig. S7.** Basal and *Drm*-capa-1–stimulated fluid transport rates were measured in isolated tubules from *capa*-knockdown flies and parental controls. Data are expressed as mean fluid transport rate (in nanoliters per minute) ± SEM, $n = 6–10$. No significant differences for the three genotypes were observed in basal or stimulated rates (Student's *t* test).

**Table S1. Model effect test from GLM fitting cold shock survival as a function of sex, treatment (cold shock or RCH), line (*capa>capa RNAi*, *capa/+*, *capa RNAi/+*), and an interaction between sex and treatment**

| Term | $\chi^2$ value | Df | *P* value |
|---|---|---|---|
| (Intercept) | 15.5525 | 1 | 8.03E-05 |
| Sex | 11.4937 | 1 | 0.000698 |
| Treatment | 39.6008 | 1 | 3.12E-10 |
| Line | 2.1566 | 2 | 0.340181 |
| Sex*treatment | 8.0591 | 1 | 0.004528 |

We fit a GLMM using the glmer function in R, assuming a binomial distribution and a logit link function. Starting with a saturated model, the Akaike information criterion was used to select the best-fitting model, and the effects of those terms are displayed in the table. Df, degrees of freedom.

**Table S2. Model effect test from Cox proportional hazards model fitting chill coma time as a function of exposure time, line (*capa>capa RNAi*, *capa/+*, *capa RNAi/+*), and their interaction**

| Term | $\chi^2$ value | Df | *P* value |
|---|---|---|---|
| Exposure time | 310.7053 | 1 | <1E-20 |
| Line | 16.41521 | 2 | 0.000273 |
| Exposure time*group | 0.895329 | 2 | 0.639119 |

A Cox proportional hazards model was fit in JMP with CCR as the response variable.

**Table S3. Risk ratios test for CCR across lines**

| Line 1 | Line 2 | Risk ratio | *P* value |
|---|---|---|---|
| *capa RNAi/+* | *capa>capa RNAi* | 1.519288 | 0.000502 |
| *capa/+* | *capa>capa RNAi* | 1.490212 | 0.000712 |
| *capa/+* | *capa RNAi/+* | 0.980862 | 0.870439 |

The results show that the *capa*-knockdown flies tended to have longer recovery times across the range of exposure times, whereas the two control lines did not differ from each other.

**Table S4. Primer sequences used in this study**

| Gene | Direction | Sequence 5′ to 3′ |
|---|---|---|
| *Capa$^1$-Gal4* | F | GCGGTACCCTCAAGTACCACGTTTCTGC |
| | R | GCGGTACCTGCAAGACTATTTAGGACGA |
| *D. melanogaster rp49* | F | ATCCGGCCAGCATACAG |
| | R | TCCGACCAGGTTACAAGAA |
| *D. mojavensis capa* | F | CCGAGCTGAGAAAGTGGGCCC |
| | R | CGCTTGCCCAGACGTGGTCCG |
| *D. mojavensis rp49* | F | GTCGTCGCTTCAAGGGCCAAT |
| | R | ATGGGCGATCTCACCGCAGTA |
| *D. montana capa* | F | CGCAGCGATCCGAGTCTGGTG |
| | R | GGTGCGTTTGTCCAGCGCCTG |
| *D. montana rp49* | F | AGCACTTCATCCGTCACCAGTC |
| | R | CGCACTCTGTTGTCAATACCCTTG |
| *A. aegypti capa* | F | TATCATCCGACCTGGACAG |
| | R | GAGGGGTGTAGACTTCAGTTC |
| *A. aegypti RpS17* | F | AGACAACTACGTGCCGGAAG |
| | R | TTGGTGACCTGGACAACGATG |
| *A. gambiae capa* | F | CGTCGGACTGTTTGCGTTTC |
| | R | GCTTCATCTCCTTCATCGGGTAG |
| *A. gambiae RpS7* | F | GGCGATCATCATCTACGTGC |
| | R | GTAGCTGCTGCAAACTTCGG |