

11-2001

The Effect of Thermal Stress on the Mating Behavior of Three *Drosophila* Species

Zachary J. Patton
Cleveland State University

Robert A. Krebs
Cleveland State University, r.krebs@csuohio.edu

Follow this and additional works at: https://engagedscholarship.csuohio.edu/scibges_facpub

 Part of the [Biology Commons](#)

How does access to this work benefit you? Let us know!

Publisher's Statement

© 2001 by The University of Chicago

Recommended Citation

Patton, Z. J., & Krebs, R. A. (2001). The effect of thermal stress on the mating behavior of three *Drosophila* species. *Physiological And Biochemical Zoology*: PBZ, 74(6), 783-788.

This Article is brought to you for free and open access by the Biological, Geological, and Environmental Sciences Department at EngagedScholarship@CSU. It has been accepted for inclusion in Biological, Geological, and Environmental Faculty Publications by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

The Effect of Thermal Stress on the Mating Behavior of Three *Drosophila* Species

Zachary J. Patton

Robert A. Krebs*

Department of Biological, Geological, and Environmental Sciences, Cleveland State University, Cleveland, Ohio 44115-2403

Accepted 6/21/01

ABSTRACT

Selection may act on the weakest link in fitness to change how a species adapts to an environmental stress. For many species, this limitation may be reproduction. After adult *Drosophila melanogaster*, *Drosophila simulans*, and *Drosophila mojavensis* males were exposed to varying levels of thermal stress well below those that endanger life, courtship and mating frequency declined. The regression coefficients of both courtship and mating success did not differ significantly between *D. melanogaster* and *D. simulans* males. In contrast, significant differences were present between the two cosmopolitan species and *D. mojavensis*. Courtship frequency decreased at a much slower rate in *D. mojavensis* than in *D. melanogaster* and *D. simulans*, and while heat-stressed *D. mojavensis* males continued to court, many did not mate. In the cosmopolitan species, courting males almost always mated successfully. Courtship behaviors, including wing waving, were observed in *D. mojavensis* at temperatures that prohibited flight, while flight, courtship, and mating were knocked out simultaneously in *D. melanogaster*. One possible explanation for decreased flight ability and courtship success may be the reduced heat shock response in the flight muscle tissue because Hsp70 expression was lowest in the thoracic tissue of both *D. melanogaster* and *D. mojavensis*.

Introduction

Experiments that have tested thermotolerance in *Drosophila* mainly evaluate performance characters such as survival, knockdown, fertility, and fecundity (Huey et al. 1992; Krebs and Loeschcke 1994; Gilchrist and Huey 1999). While each of

these techniques provides useful information about the effects of thermal stress on fitness, mating behavior may likely be affected sooner than survivorship by thermal stress will and therefore may better demonstrate initial fitness effects of thermal stress in adult *Drosophila*.

This study examined variation in behavioral responses to high temperatures in three well-known species (*Drosophila melanogaster*, *Drosophila simulans*, and *Drosophila mojavensis*), primarily because of their known differences in survival after heat stress (Krebs 1999). *Drosophila melanogaster* and *D. simulans* are sympatric cosmopolitan species that are endemic to temperate regions, feeding on necrotic fruit. *Drosophila mojavensis* is a cactophilic species that is specifically found on the rotten arms of agria (*Stenocereus gummosus*), California barrel (*Ferocactus acanthodes*), and organ pipe (*Stenocereus thurberi*) cacti (Heed and Mangan 1986). We compared the upper performance curves (Huey and Stevenson 1979) of courtship and mating among desert and cosmopolitan species for differences in performance breadth. Since the desert flies encounter the most extreme thermal environment of those species analyzed here, and they have little or no refuge from thermal stress (A. G. Gibbs, M. C. Perkins, and T. A. Markow, unpublished data), selection should favor increased courtship and mating ability after stress and thus an increased performance breadth in *D. mojavensis*.

Finally, we examined variation in Hsp70 expression among tissues of the head, thorax, and abdomen. Since *Drosophila* courtship behavior is made up of numerous components that include wing waving (Markow and Hanson 1981), thermal damage to the flight muscle may impair courtship and thus create negative consequences to male fitness. Low Hsp70 expression has been correlated with damage in larval tissue (Krebs and Feder 1997), and, therefore, tests of Hsp70 levels in the flight muscle may indicate whether this is one of the first tissues to be negatively affected by thermal stress in adult flies.

Material and Methods

Rearing Protocol

Drosophila melanogaster and *Drosophila simulans* were obtained in September of 1998 at Patterson Farms in Chesterland, Ohio. A number of rotten apples were collected from the orchard and were subsequently placed in rearing chambers. Flies that emerged in the rearing chambers were separated by sex under CO₂ anesthesia, females were placed separately in vials, and emerging offspring were identified by species. The *Drosophila*

* Corresponding author; e-mail: r.krebs@csuohio.edu.

mojavensis were obtained from Teri Markow (University of Arizona strain SOSCO297), who directly aspirated adult flies off of a rotting organ pipe cactus located in the Sonoran Desert near the town of San Carlos in the state of Sonora, Mexico.

Flies for all of the experiments were reared in half-pint glass bottles on a food medium consisting of cornmeal, yeast, molasses, agar, tegosept, and propionic acid. The medium in each rearing bottle was lightly sprinkled with dry yeast before placing 20–30 adult flies in each bottle. Flies were allowed to mate and lay eggs for 3–4 d and were then transferred into new bottles. After 9 d (or three transfers), the parent flies were discarded and new flies were drawn from the population to maintain genetic diversity. All flies were reared in thermally controlled incubators with a 12L : 12D cycle. Rearing temperatures for *D. melanogaster* and *D. simulans* were 21°C L : 18°C D, while *D. mojavensis* was reared at 27°C L : 21°C D.

For *D. melanogaster* and *D. simulans*, rearing bottles were cleared of flies at approximately 0730 hours, and virgin flies were subsequently collected at 1600 hours. *Drosophila mojavensis* virgins were simply collected once a day since they do not begin mating until more than 24 h after emergence (Markow 1982). All species were then anesthetized using CO₂ and were separated by sex under a dissection microscope. Male and female flies were placed separately into glass vials (15 males or 10 females per vial) containing approximately 2 mL of cornmeal medium and a sprinkling of dry yeast. *Drosophila melanogaster* and *D. simulans* males and females were allowed to mature for 4–5 d before use in experiments, and *D. mojavensis* males and females were allowed to mature for 7–8 d before use. The primary reason for the difference in maturation time is that young mature virgin flies were desired for the experiments, and adults of *D. mojavensis* mature more slowly (Markow 1982). During maturation, the flies were kept in incubators with the same settings as for rearing.

Mating Experiments

The mating experiments were carried out in the same general fashion for all three species. After the flies had matured, the males were subjected to thermal stress for 1-h periods usually between the hours of 0800 and 0900. This was done by placing vials containing 15 males into a hot water bath ($\pm 0.1^\circ\text{C}$; Polyscience). The vials were sealed using moistened rubber stoppers over cotton balls, which ensured that internal humidity remained high, and the vials were inverted to prevent flies from becoming stuck in the warm medium. For each temperature treatment, 10 males were chosen at random and paired individually with nonstressed females. A typical experiment contained three stress temperatures and a nonstressed control group. Mating behaviors, specifically the ability to court and the ability to mate, were measured during the hour immediately following treatment, and treatment began within 1 h after photophase in the morning. Courtship was counted when any typ-

ical male courtship behavior such as chasing, orienting, or wing waving was observed. Mating was counted when males successfully mounted the females.

There were five different temperature treatments for *D. melanogaster* males (36°, 36.5°, 37°, 37.5°, and 38°C) and for *D. simulans* males (34.5°, 35°, 35.5°, 36°, and 36.5°C). *Drosophila mojavensis* males were treated at 36.5°, 37°, 37.5°, 38°, 38.5°, and 39°C. The control males for all three groups were maintained at room temperature throughout the experiments. The temperature treatments were chosen because they are similar to extremes that could be experienced in nature (Feder 1996; A. G. Gibbs, M. C. Perkins, and T. A. Markow, unpublished data).

Flight Experiments

Virgin male flies were collected as described above and were placed in vials ($n = 10$ per vial) containing approximately 2 mL of medium and a sprinkle of dry yeast. *Drosophila melanogaster* males were treated for 1 h in a hot water bath at 35°, 36°, 37°, or 38°C, while *D. mojavensis* males were treated at 36°, 37°, 38°, 38.5°, or 39°C. The flies were then placed on white paper, and we scored the proportion that flew away. Males that did not fly immediately were gently prodded with a soft paintbrush.

Hsp70 Analysis

The mature *D. melanogaster* males were exposed to 36°C for 1 h and were then allowed to recover at room temperature (22°C) for an equal amount of time. The same procedure was followed for *D. mojavensis* except that the treatment temperature was 39°C, which is the temperature that maximally induces expression in each species (Krebs 1999). Both species were then put on ice to ease dissection. Heads, thoraces, and abdomens were separated on ice and stored in groups of five in centrifuge tubes that contained 10 mL of 0.9% phosphate-buffered saline. Each completed tube was placed in liquid nitrogen and later transferred to a -80°C freezer until needed. Samples were then subjected to enzyme-linked immunosorbent assay for Hsp70 (ELISA) that produces a reaction that is proportional to the target protein concentration in the sample (Welte et al. 1993; Feder et al. 1996).

Statistical Analysis

Data were analyzed using the Systat 8.0 (Wilkinson 2001) statistical-analysis package and data analysis software in Excel (Microsoft). Courtship and mating data (Fig. 1) were tested for polynomial effects using multiple regression and were put into a general linear model (GLM) to compare the temperature by species interactions among *D. melanogaster*, *D. simulans*, and *D. mojavensis*.

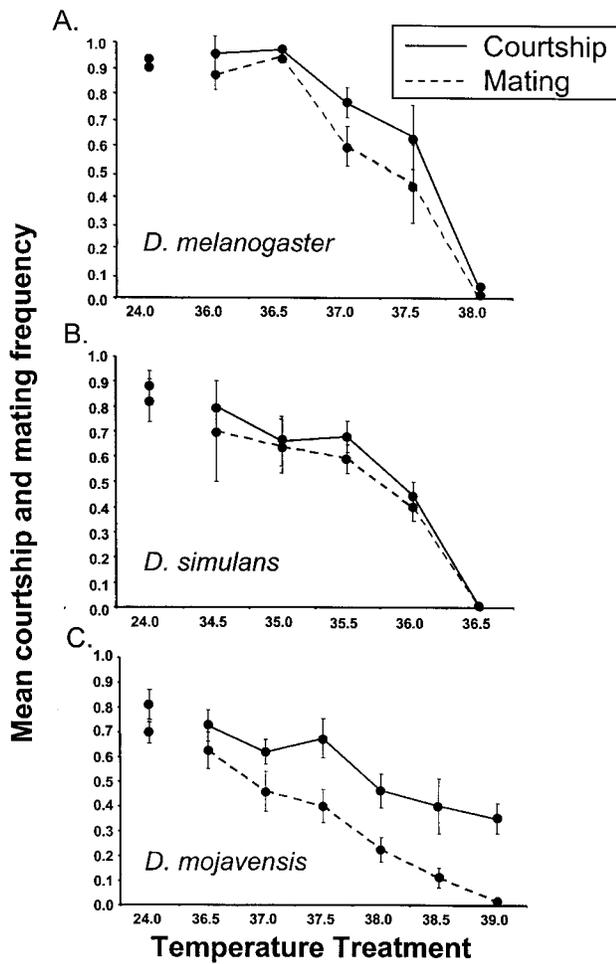


Figure 1. The frequency of courtship and mating in (A) *Drosophila melanogaster*, (B) *Drosophila simulans*, and (C) *Drosophila mojavensis* males after 1 h of thermal stress at a range of high temperatures. Control groups tested at room temperature (24°C). Means are made up of five to nine replicates of 10 mating pairs ± 1 SE.

Results

Courtship and Mating Experiments

Courtship and mating fell off steeply as temperatures increased above those that allow optimal performance (Fig. 1). Performance of all three fly species dropped in frequency from nearly one to nearly zero over a range of approximately 2°C, and tests of polynomial effects were found not to be significantly different from zero for any of these species. For this reason, the data were treated as linear.

Drosophila melanogaster continued to court and mate at higher temperatures than did *Drosophila simulans*, which was expected because these species were chosen because of their marked differences in adult thermotolerance. However, the

slope of the linear extreme end of the respective performance curves did not differ between these two species (Table 1).

Drosophila mojavensis also continued to court and mate at much higher temperatures than either of the others. This species, however, differed significantly from both *D. melanogaster* and *D. simulans* with respect to the slopes of the performance curves (Table 1).

Unlike results for *D. melanogaster* and *D. simulans*, the slopes of courtship and mating performance curves in *D. mojavensis* differed significantly ($P < 0.01$ GLM; Fig. 1). Mean mating frequency at 39°C was 0.02 in *D. mojavensis*, while mean courtship frequency at the same temperature was 0.35.

Test for Flight Capability

As expected for a more thermotolerant species, *D. mojavensis* flew after exposure to higher temperatures than *D. melanogaster* (Fig. 2). Unlike the courtship and mating results, where *D. mojavensis* showed a slower decrease in performance than *D. melanogaster*, flight ability for males in these two species declined similarly.

Test for Hsp70 Expression in Flight Muscle

ELISA was used to compare Hsp70-expression levels in the head, abdomen, and thorax of *D. melanogaster* and *D. mojavensis* (Fig. 3). Thoracic tissue produced significantly less Hsp70 than did tissue of either the head or the abdomen. Differences in Hsp70 expression among *D. melanogaster* and *D. mojavensis* were large but proportionally the same, and, therefore, normalizing the data gave no significant species by body part effect. Hsp70 concentration was expressed relative to that in a standard homogenate of heat-treated *D. melanogaster*.

Table 1: Regression coefficients for effect of temperature on *Drosophila* courtship and mating frequency (in bold) and matrix of *P* values for pairwise tests of temperature by species interactions

	<i>Drosophila melanogaster</i>		<i>Drosophila simulans</i>		<i>Drosophila mojavensis</i>	
	Court	Mate	Court	Mate	Court	Mate
<i>D. melanogaster</i>	-.46	-.43				
<i>D. simulans</i>	NS	NS	-.40	-.37		
<i>D. mojavensis</i>	.00	.01	.00	.06	-.16	-.22

Note. Values determined from GLM (general linear model). This statistic tests whether the slopes of the linear regressions differ significantly. Temperatures used to calculate the regression coefficients were 36°–37.5°C for *D. melanogaster*, 34.5°–36°C for *D. simulans*, and 36.5°–38.5°C for *D. mojavensis*.

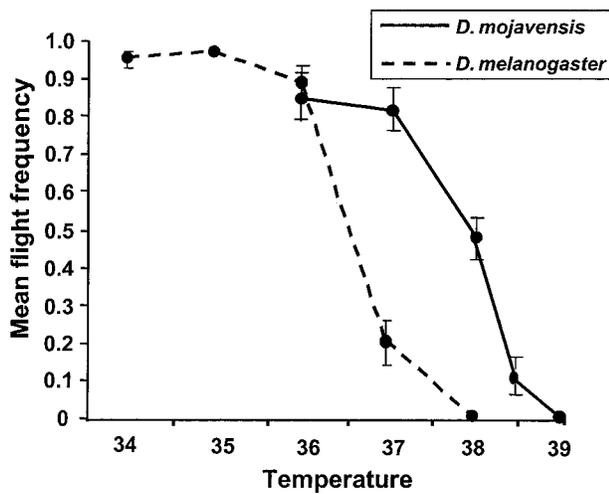


Figure 2. Flight capability of male *Drosophila* after 1 h of thermal stress. Means are made up of four to five replicates of 10 flies per replicate \pm 1 SE.

Discussion

Thermal Shifts in Courtship and Mating Success

Males of *Drosophila mojavensis* (which come from a desert environment) continue to court and to mate at much higher temperatures than do males of either *Drosophila melanogaster* or *Drosophila simulans*, the two cosmopolitan and temperate species. Indeed, these three species were chosen for study specifically because they vary in thermotolerance. *Drosophila simulans* is the most sensitive to temperature treatments for adult survival, followed by *D. melanogaster* and *D. mojavensis* (Krebs 1999). Stratman and Markow (1999) also indicated that *D. mojavensis* survives at much higher temperatures than *D. simulans*.

Differences in courtship and mating frequency between *D. melanogaster* and *D. simulans* show a simple shift in thermotolerance that has several plausible explanations beyond the possibility of an adaptation to changing environmental conditions. Although sympatric over much of their distribution, *D. simulans* is more sensitive to high temperature and low relative humidity than is *D. melanogaster* (Parsons 1978), yet it often outnumbers its sympatric counterpart. This variation in relative abundance shifts from where conditions are favorable to environmental conditions that vary. Under more stable environmental conditions, *D. simulans* is more abundant than is *D. melanogaster*, while *D. melanogaster* tends to replace *D. simulans* in a variable environment (Parsons 1978). Perhaps the greater success of *D. simulans* adults during favorable environmental conditions comes at the cost of reduced stress resistance. In contrast, the increased performance of *D. melanogaster* during stressful conditions may make this species less competitive

in conditions that favor *D. simulans*, which, in terms of ectotherm performance, suggests a trade-off (Hoffmann 1995). *Drosophila melanogaster* may have a slightly lower optimal performance but a greater performance breadth than *D. simulans*, while *D. simulans* may have a higher optimum performance and a more narrow performance breadth than *D. melanogaster*. Alternatively, *D. melanogaster* and *D. simulans* adults may face little selection for increased performance at high temperatures because they avoid stressful temperatures by locating favorable microhabitats (Feder 1996). Therefore, variation in adult thermotolerance may be an artifact of selection for increased stress resistance in other life stages. This is probably not the case in *D. mojavensis* because adults and larvae may experience the same thermal environment consistently (A. G. Gibbs, M. C. Perkins, and T. A. Markow, unpublished data).

Because the area of the performance curve analyzed includes only temperatures that decrease performance, we hypothesized that the change in courtship and mating at high temperatures would be linear and rapid. The performance curves support this hypothesis in that both courtship and mating fell to zero in 2°C or less in *D. melanogaster* and *D. simulans*, but these curves for *D. mojavensis* declined much less rapidly. These effects on mating behavior also differed from that on survival (Krebs 1999; Stratman and Markow 1999) and flight, which decreased in a similar fashion in all three species. Therefore, the performance curves for courtship and mating changed shape in the desert species, *D. mojavensis*; either the overall breadth of the performance curve has increased or the upper-temperature tolerance has broadened, as predicted by Huey and Kingsolver (1993) for desert species. Either way, many indi-

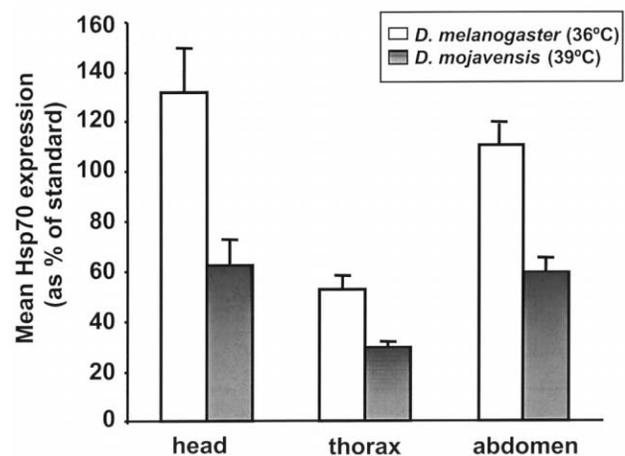


Figure 3. Mean Hsp70 expression levels in the head, thorax, and abdomen of male flies exposed to 1 h of a thermal stress that maximizes expression (\pm 1 SE). Hsp70 is presented as a percent of standard from ELISA.

viduals were able to court and to mate after exposure to higher temperatures than predicted.

In these experiments, the duration and intensity of heat shock was short, as was the observation period for courtship. Thus, we tested how heat interfered with mating in the short term. Many males began courting females soon after the observation period was over, and with a lengthy recovery, males will eventually court even after exposure to a nearly lethal stress (Krebs and Loeschcke 1996). That such a mild thermal stress can reduce courtship and mating indicates that behavioral change may better represent fitness consequences of stress than will survival, the variable most often used in stress analysis (Feder and Hofmann 1999). Preliminary results suggest that all males who mate pass viable sperm (Patton 2000).

Relationship among Courtship, Mating, and Flight Behavior

Like survival, flight tests indicated no significant differences between *D. mojavensis* and *D. melanogaster* with respect to the shape of the performance curve, although *D. mojavensis* can fly after exposure to higher temperatures. Therefore, flight in *D. mojavensis* did not respond in a similar fashion to thermal stress as did courtship and mating. However, we expected that flight, courtship, and mating data would be similar since males employ wing waving in courtship (Ewing and Bennet-Clark 1968; Spiess 1987). Observations during mating experiments indicate that some *D. melanogaster* court successfully without the wing waving display, although the *D. melanogaster* courtship display is quite complex and visual cues play a large role (Markow and Hanson 1981). Therefore, males may provide adequate information to females even with impaired wings.

One final and important difference between the thermal response of *D. mojavensis* and *D. melanogaster* is that 40% of *D. mojavensis* males continue to court after exposure to 39°C, when mating is unlikely. Perhaps females cannot recognize courting males, or they reject the courtship displayed by males. Markow and Toolson (1990) found that heat stress may change the composition of epicuticular hydrocarbons in *D. mojavensis* males, making them appear more female and thus less attractive to potential mates. Alternatively, if flight muscle sustains damage from thermal stress, a direct outcome would be to change the effectiveness of wing waving displays and song during courtship. *Drosophila mojavensis* males readily court using their wings after exposure to temperatures that eliminate flight.

Damage to the flight muscle is suggested by analysis of Hsp70 expression. The thorax expresses a reduced heat shock response compared to that in the head and abdomen of both *D. melanogaster* and *D. mojavensis*. In *D. melanogaster*, larval tissue with low Hsp70 expression following thermal stress is the most susceptible to thermal damage, a result that Krebs and Feder (1997) explained as a trade-off. That is, Hsp70 may interfere with metabolism and nutrient uptake in the midgut, and the heat shock response may consequently be repressed in this

tissue, leaving it susceptible to thermal damage. Perhaps a similar outcome exists in the flight muscle of adult *Drosophila*. Hsp70 may interfere with the complex metabolic activity needed to sustain flight and coordinate the wing waving display. Denlinger et al. (1991) have demonstrated tissue-specific variation in Hsp expression in the flesh fly *Sarcophagidae crassipalpis*, in which flight muscle does not express Hsp72 (the heat shock protein found in the brain and integument after thermal stress). Instead, Hsp65, a related heat shock protein, is expressed in this tissue following thermal stress. Perhaps this switch in expressed Hsp's represents a solution to thermal stress in the flight muscle of *S. crassipalpis* that is analogous to reduced expression of Hsp70 in the flight muscle of *D. mojavensis*. Although these physiological changes are clear, additional comparative studies are required to test whether the specific changes reported here are actual adaptations to ecological change rather than a by-product of the many potential influences that the thermal environment can impart on *Drosophila* (Dahlggaard et al. 2001).

Acknowledgments

We thank Paul Doerder and Tarun Mal for valuable suggestions and advice given to Z.J.P. and Allen Gibbs for his review of the manuscript.

Literature Cited

- Dahlggaard J., E. Hasson, and V. Loeschcke. 2001. Behavioral differentiation in oviposition activity in *Drosophila buzzatii* from highland and lowland populations in Argentina: plasticity or thermal adaptation? *Evolution* 55:738–747.
- Denlinger D.L., K.H. Joplin, C.-P. Chen, and R.E. Lee, Jr. 1991. Cold and heat shock. Pp. 131–148 in R.E. Lee and D.L. Denlinger, eds. *Insects at Low Temperature*. Chapman & Hall, New York.
- Ewing A.W. and H.C. Bennet-Clark. 1968. The courtship songs of *Drosophila*. *Behaviour* 31:288–301.
- Feder M.E. 1996. Ecological and evolutionary physiology of stress proteins and the stress response: the *Drosophila melanogaster* model. Pp. 79–102 in I.A. Johnston and A.F. Bennet, eds. *Phenotypic and Evolutionary Adaptation to Temperature*. Cambridge University Press, Cambridge.
- Feder M.E., N.V. Cartaño, L. Milos, R.A. Krebs, and S.L. Lindquist. 1996. Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. *J Exp Biol* 199:1837–1844.
- Feder M.E. and G.E. Hofmann. 1999. Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 61:243–282.
- Gilchrist G.W. and R.B. Huey. 1999. The direct response of

- Drosophila melanogaster* to selection on knockdown temperature. *Heredity* 83:15–29.
- Heed W.B. and R.L. Mangan. 1986. Ecology and genetics of Sonoran Desert *Drosophila*. Pp. 311–345 in M. Ashburner, H.L. Carson, and J.N. Thompson, Jr., eds. *The Genetics and Biology of Drosophila*. Academic Press, London.
- Hoffmann A.A. 1995. Acclimation: increasing survival at a cost. *Trends Ecol Evol* 10:1–2.
- Huey R.B., W.D. Crill, J.G. Kingsolver, and K.E. Weber. 1992. A method for rapid measurement of heat and cold resistance of small insects. *Funct Ecol* 6:489–494.
- Huey R.B. and J.G. Kingsolver. 1993. Evolution of resistance to high temperature in ectotherms. *Am Nat* 142(suppl.): S21–S46.
- Huey R.B. and R.D. Stevensen. 1979. Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. *Am Zool* 19:357–366.
- Krebs R.A. 1999. A comparison of Hsp70 expression and thermotolerance in adults and larvae of three *Drosophila* species. *Cell Stress Chaperones* 4:234–249.
- Krebs R.A. and M.E. Feder. 1997. Tissue specific variation in Hsp70 expression and thermal damage in *Drosophila melanogaster* larvae. *J Exp Biol* 200:2007–2015.
- Krebs R.A. and V. Loeschcke. 1994. Effects of exposure to short term heat stress on fitness components in *Drosophila melanogaster*. *J Evol Biol* 7:39–49.
- . 1996. Acclimation and selection for increased resistance to thermal stress in *Drosophila buzzatii*. *Genetics* 142: 471–479.
- Markow T.A. 1982. Mating systems of cactophilic *Drosophila*. Pp. 273–287 in J.S.F. Barker and W.T. Starmer, eds. *Ecological Genetics and Evolution*. Academic Press, Sydney.
- Markow T.A. and S.J. Hanson. 1981. Multivariate analysis of *Drosophila* courtship. *Proc Natl Acad Sci USA* 78:430–434.
- Markow T.A. and E.C. Toolson. 1990. Temperature effects on epicuticular hydrocarbons and sexual isolation in *Drosophila mojavensis*. Pp. 315–331 in J.S.F. Barker, W.T. Stamer, and R.J. MacIntyre, eds. *Ecological and Evolutionary Genetics of Drosophila*. Plenum, New York.
- Parsons P.A. 1978. Resistance of the sibling species *D. melanogaster* and *D. simulans* to high temperatures in relation to humidity: evolutionary implications. *Evolution* 33:131–136.
- Patton Z.J. 2000. The effect of thermal stress on the mating behavior of three *Drosophila* species. MS thesis. Cleveland State University.
- Spiess E.B. 1987. Discrimination among prospective mates in *Drosophila*. Pp. 75–117 in D.J.C. Fletcher and C.D. Michener, eds. *Kin Recognition in Animals*. Wiley, New York.
- Stratman R. and T.A. Markow. 1999. Resistance to thermal stress in desert *Drosophila*. *Funct Ecol* 12:965–970.
- Welte M.A., J.M. Tetrault, R.P. Dellavalle, and S.L. Lindquist. 1993. A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. *Curr Biol* 3:842–853.
- Wilkinson L. 1998. SYSTAT for Windows: Statistics. Version 8.0. SYSTAT, Evanston, Ill.

Copyright of *Physiological & Biochemical Zoology* is the property of University of Chicago Press and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.