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TECHNICAL REPORT

A method for rapid measurement of heat or cold resistance of small insects

R. B. HUEY, W. D. CRILL, J. G. KINGSOLVER and K. E. WEBER*

*Department of Zoology NJ-15, University of Washington, Seattle, Washington 98195 and *Department of Biological Sciences, University of Southern Maine, Portland, Maine 04103, USA*

Summary

1. We describe a new technique for rapidly measuring the heat or cold resistance of many small insects. We heat (or cool) insects in a temperature-controlled column and measure the temperature at which they are knocked down.

2. An artificial selection experiment on heat resistance demonstrates that knock-down temperatures respond rapidly to selection. After only four generations of selection, the experimental and control lines differed significantly in both knock-down temperature and physiological heat tolerance, as measured by per cent survival of a heat shock.

3. Potential applications (acclimation, ageing, selection) and technical problems (body size, humidity, statistical considerations) are evaluated.

Key-words: Drosophila, mass selection, thermal sensitivity

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Introduction

Measures of tolerance to extreme heat or cold are widely used in physiological ecology and in evolutionary physiology (Cowles & Bogert 1944; Paladino *et al.* 1980; Prosser 1986; Hoffmann & Parsons 1991). These delimit the range of body temperatures that species or populations can survive (Fry 1957); and, to the extent that they correlate with 'optimal' performance temperatures (Becker & Genoway 1979; Garland, Huey & Bennett 1991), they serve also as convenient and ecologically relevant indices of overall thermal sensitivity (Huey 1982; Hoffmann & Parsons 1991).

In studies with lower vertebrates and some large insects (Heath & Wilkin 1970; Becker & Genoway 1979; Paladino *et al.* 1980); tolerance to extreme temperature is often assessed by heating (or cooling) an animal until it loses its righting response (Cowles & Bogert 1944) or goes into spasms (Hutchison 1961). These 'critical thermal maximum' ('minimum') temperatures are easily measured, and the tested animals usually survive.

In studies with small insects, however, critical body temperatures cannot be measured directly because such insects are too small (Krogh 1948). Consequently, heat tolerance of small insects is usually measured by exposing groups to a high (or low) ambient temperature for a set period [e.g. 39·5 °C for 0·5 h (Levins 1969; White, DeBach & Garber 1970; Coyne, Bundgaard & Prout 1983) or −3·0 °C for 24 h (Davidson 1990)], waiting 24 h, and then determining the percentage of individuals that survived. If percentage survival is measured over a range of temperatures or times of exposure, a LD_{50} can be estimated (Kimura 1988; Quintana & Prevosti 1990). Nevertheless, these widely used techniques have significant disadvantages: many test animals are killed and the survivors are sometimes sterilized (David *et al.* 1983; but see White *et al.* 1970; Parsons 1980). Consequently, per cent survival techniques may be unsuitable for studies of quantitative genetics or of responses to artificial selection, unless indirect 'family' analyses are used (Morrison & Milkman 1978; Kilias & Alahiotis 1985; Quintana & Prevosti 1990). Moreover, repeatability can be low (Coyne *et al.* 1983; Huey, Partridge & Fowler 1991); and the technique yields populational rather than individual statistics, so that statistical power is reduced.

We have developed an efficient new way to measure the temperature tolerance of many small insects (e.g. parasitic wasps, *Drosophila*). We measure temperatures ('knock-down temperatures' $=T_{kd}$) at which such insects are incapacitated by acute exposure to heat or cold. These temperatures correspond to the 'critical' temperatures measured in lower vertebrates (Paladino *et al.* 1980). In studies with *D. melanogaster* Meigen, we simultaneously measure knock-down temperatures on large numbers of flies (1000 or more) and have found that

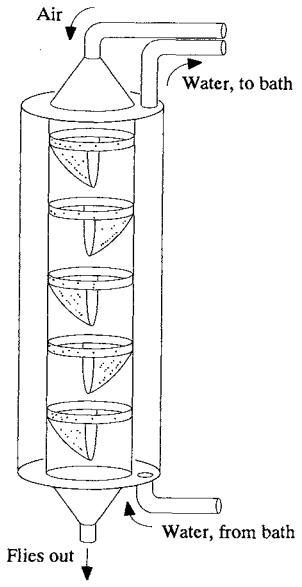

Fig. 1. Apparatus for measuring and 'fractionating' small arthropods by knock-down resistance to heat or cold. The study animals are first put into the top of the column, and the temperature within the column then warmed (or cooled) by pumping water (ethylene glycol) from a temperature-controlled bath through the surrounding water jacket. When the animals reach an incapacitating body temperature (knock-down temperature), they fall out of the column into collecting vials below. Knock-down temperatures of small insects are closely approximated (see Appendix) by measuring air temperature within the column.

measurement does not affect the viability or fertility of the flies. Thus this technique should facilitate quantitative genetic and selection experiments. Moreover, it should be useful in diverse studies of the thermal biology of small arthropods. Here we describe the apparatus and experimental protocols involved in measuring knock-down temperatures and discuss pilot applications and potential limitations.

Materials and methods

We index heat (or cold) resistance as the temperature at which insects are incapacitated by extreme heat (or cold). In brief, the insects are heated (or cooled) at a controlled rate in a water-jacketed glass column. At some limiting temperature insects become incapacitated and fall out of the bottom of the column into collecting tubes. Because small insects have short thermal time constants (Stevenson 1985), their body temperature will closely track ambient air temperatures in the column (Appendix). Consequently, by monitoring the ambient temperature inside the column and by changing collecting tubes at predetermined intervals of temperature change (e.g. 0·5°C), we rapidly and simultaneously measure the knock-down temperatures for large numbers of insects, and the insects are automatically fractionated (i.e. separated into phenotypic classes) by knock-down temperatures.

HEAT RESISTANCE

To measure knock-down temperatures, we have modified an apparatus developed originally (Weber 1988; Weber & Diggins 1990; see also Cohan & Graf 1985; Hoffmann & Cohan 1987) to study knock-down resistance of flies to ethanol and other vapours. The column consists of a water-jacketed, glass cylinder 120×7·5cm, with internal baffles (Fig. 1; details in Weber 1988). In our pilot studies with *Drosophila*, we initially set the temperature of the column at 30°C, a warm but not disabling temperature. Flies are then introduced at the top of the column, where they tend to remain (they are negatively geotropic). Fine-mesh baffles in the column provide extensive perches for the flies and increase the efficiency of fractionation (Weber 1988), while allowing air currents (see below) to pass through the column with only limited resistance. The temperature of the water jacket is then raised at a set rate (*c.* 0·5°C min^{-1}) by heating water in the adjacent bath, and rapidly circulating water through the water jacket. The increasingly warm water in the jacket heats the air and flies inside the column. When flies reach their critical temperature, they fall through the baffles, out through a fluon-coated funnel at the bottom of the column, and into a narrow fluon-coated collecting tube (at room temperature) from which they cannot escape. We change collecting tubes every 0·5°C and subsequently sex and count the flies in each tube. [If sexing is not required, falling insects can efficiently be counted electronically by placing a photocell detector between the column and the collecting tubes (Weber 1988).] Data from a sample run are shown in Fig. 2.

To reduce thermal gradients within the column (e.g. top to bottom, wall to centre) and to reduce the thermal time constant of the flies (see below), we pump air (equilibrated to the temperature of the water jacket) through the column from top to bottom. [Incurrent air is passed first through a copper

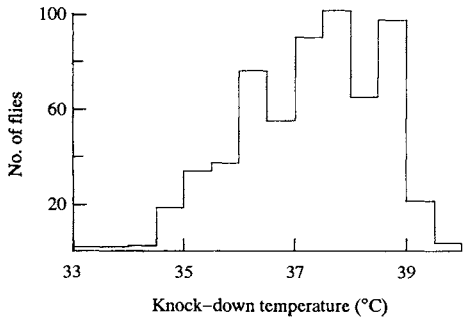

Fig. 2. A histogram of frequency of upper knock-down temperatures for a sample (n=602) of male *Drosophila melanogaster*.

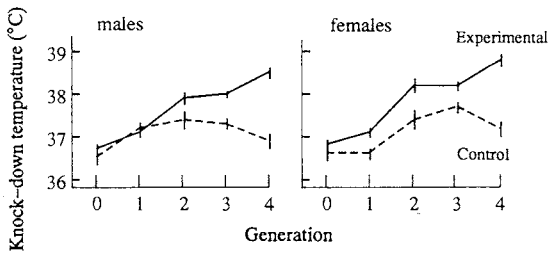

Fig. 3. Mean knock-down temperatures (±1SE) of males from selected and control lines of *Drosophila melanogaster* at generations 0 through 4 of selection (top 25%) on knock-down resistance to heat. Knock-down temperatures (T_{kd}) responded rapidly to selection, such that T_{kd} of the selected line increased by about 1·5°C relative to that of the control line.

coil in the water-bath and then runs counter-current beside the excurrent water line and inside a common insulative sheath.] Air flow can be adjusted to minimize intracolumn gradients. At low flow rates (0·5 litres min^{-1}), the time constant of insects the size of *D. melanogaster* is about 20s.

Fractionation is fast: in approximately 20 min, we can fractionate over 1000 flies according to their critical temperature. Consequently, rapid acclimation (Czajka & Lee 1990), heat-shock responses (Huey & Bennett 1990), or desiccation (Maynard Smith 1956) should not confound our measurements. However, humidified or dehumidified air can be passed through the column, if appropriate (see below).

Alternatively, the water jacket could be set at a constant high (or low) temperature and then the length of *time* measured before the insects were knocked down. The resulting data would be directly equivalent to those in studies of resistance to ethanol vapours (e.g. Weber 1988). However, if times are long, this measure of heat resistance might be confounded by rapid acclimation (Czajka & Lee 1990).

COLD RESISTANCE

To measure lower knock-down temperatures, we replace the water in the system with ethylene glycol, and use a cooling coil in the water-bath to lower the temperature from 15°C until the flies fall out of the column. Unless the cooling coil is powerful enough to cool the column quickly, rapid acclimation (Czajka & Lee 1990) could confound the measurements. Cooling rates can also be increased by placing the entire apparatus in a cold room and by reducing the fluid volume (i.e. using a small column and water-bath). To prevent water (which can trap flies) from condensing on the walls of the column during cooling, incurrent air can be passed through a desiccator on its way to the column. Because evaporation in dry air is minimal at low air temperatures, dry air is unlikely to influence estimates of lower knock-down temperatures.

Validation

Knock-down temperatures may reflect actual differences either in physiological heat (or cold) resistance or, possibly, in the behavioural willingness of flies to 'hang on' when confronted with a rapidly changing thermal environment. In the latter case the insects would be falling out at temperatures below their true critical temperatures. Two observations demonstrate that our technique does index true physiological heat or cold tolerance (i.e. ability to survive a heat or cold shock). First, flies falling out of the column are generally incapacitated and thus are not 'jumping out' while still co-ordinated. For example, flies can walk slowly and hang on in the column at 7·5°C; but when cooled to 6·5°C, they are unable to walk and start to fall out of the column in large numbers. Second, after four generations of artificial selection (described below) for high knock-down temperatures, flies from the selected line had significantly higher heat tolerance: only 5·6% of the control females survived an exposure of 39·5°C for 0·5h, whereas 36% of the selected females survived ($P<0·001$). Thus, even if selection on knock-down temperatures is selecting on behaviour, it is also selecting on true physiological tolerance.

Knock-down exposure does not influence survival or sterility of *Drosophila*. For example, less than 1% of flies in a test run died within 24h. Similarly, 11 males and 12 females were tested for fertility after being measured for upper knock-down temperature: all produced viable young.

Sample applications

Knock-down temperatures can readily characterize the heat and cold resistance of a species, population, or stock. Sample data of upper knock-down temperatures of *D. melanogaster* (originally collected from Brighton, UK; maintained for 6 years at 25°C) are shown in Fig. 2.

Knock-down temperatures can also be used to study developmental or acclimation effects on heat and cold resistance. In a pilot study (Crill 1991), for example, knock-down temperatures of *D. melanogaster* were significantly affected not only by the temperatures flies experienced during development (egg to adult, 25 *vs* 18°C), but also by the temperatures their parents experienced (25 *vs* 18°C). The effects of age or of various environmental factors on knock-down temperatures could similarly be studied.

Finally, because flies are neither killed nor sterilized by the treatment, knock-down temperatures should prove useful for quantitative genetics and for selection experiments. At the end of a four-generation experiment in which the top 25% of flies (experimental line, average $n=1251$ flies generation^{-1}) or a random 25% (control line) were used to found the next generation, the mean knock-down temperature of the experimental line had increased to 1·5°C above that of the control line, and no plateau in response was evident (Fig. 3). The increase was equivalent to about one standard deviation (phenotypic) of the control population. [The exact selection intensity is unknown. Selected males and females were kept together for 2–3 days before egg collections were made, so some remating may have occurred. Thus selection intensities were between 25 and 50%. Assuming a 50% selection intensity (no remating), the realized heritability is 28%.]

Potential limitations and technical problems

BODY SIZE

Because we assume that the air temperature in the column closely approximates insect body temperature, our technique will largely be limited to small arthropods (e.g. the size of *Drosophila*), for which time constants (τ) are very short (Stevenson 1985). For larger insects, however, the difference between T_b and T_a could be unacceptably large. Heat transfer analysis (Appendix) shows that the absolute maximum temperature difference $|T_b-T_a|$ is the product of $(b\,\tau)$, where b is the rate of heating or cooling. The temperature differential can obviously be reduced either by slowing b or by increasing air speed within the column, thus reducing τ. For large insects at reasonable flow rates, τ may still be long, and body temperature could lag significantly behind measured air temperature. However, using a microprobe thermocouple in a hypodermic needle (e.g. Heinrich 1987), one can directly measure the body temperatures of large insects as they fall out of the column.

HUMIDITY

If evaporative water loss from the insects is high (Heinrich 1979; Toolson 1987), then T_b will tend to deviate below T_a; and thus the T_a at which flies were knocked down would not be a close approximation of true T_b. However, convective heat transfer is large enough in small insects (Stevenson 1985) to oppose this, such that evaporative cooling is unlikely to be an important confounding factor. Similarly, if heating rate is slow and if evaporative water loss is very high, then insects could be knocked down by desiccation, not by heat. This problem is probably unimportant: desiccation influences survival of *Drosophila* at high temperature (Parsons 1980), but only over time scales much longer than used in our experiments (Fig. 1 in Maynard Smith 1956). In any case, if desiccation is a potential problem, humidified air can be funnelled through the column. For example, incurrent air can be humidified by bubbling it through the water-bath or water jacket. To study the effects of humidity on knock-down resistance, experiments could be run at near 0% r.h., by passing incurrent air through a desiccator. In principle, humidity could be controlled at any desired level, even during dynamic heating, by bubbling air through a computer-controlled, auxiliary water-bath. Such studies would be of interest, especially as resistance to heat and desiccation are genetically correlated in *D. melanogaster* (Hoffmann & Parsons 1989).

THERMAL GRADIENTS WITHIN THE COLUMN

Thermal gradients from top to bottom of the column can be reduced by increasing the flow rate of water in the jacket. Gradients from the wall to the centre can be reduced by narrowing the diameter of the chamber. Moreover, all gradients within the column can be minimized by increasing the flow rate of air through the chamber.

STATISTICAL CONSIDERATIONS

Because many flies are run simultaneously, knock-down temperatures for each fly in a given sample will not be statistically independent. This problem can be solved in several ways. If morphologically distinguishable species are being compared, both species can be run simultaneously through the column: any 'batch' effect is thus common to both species. If different treatment groups (e.g. acclimation groups) or populations are being compared, one or both groups could be marked (e.g. with a fluorescent dye), and again run simultaneously. In one pilot experiment, however, flies dusted with a dye had significantly higher ($P<0{\cdot}001$) knock-down temperatures than did control flies, suggesting a risk of artefacts with this approach. When either approach is impractical, several batches (temporally randomized) should be run for each group, thus enabling one to estimate the magnitude of any batch effect.

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Appendix

We are interested in how the body temperature of an insect at time t, $T_b(t)$, differs from ambient temperature in the column, $T_a(t)$, as the column is heated or cooled. Consider a simple heat flux balance where the rate of heat storage (q_{st}) is balanced by the rate of heat transfer (q_c) as a result of convection and perhaps other mechanisms. Then:

$$q_{st}=q_c$$

$$Cm\ \mathrm{d}[T_b(t)]/\mathrm{d}t=hA[T_a(t)-T_b(t)]$$

where C=the specific heat of the insect, m=body mass, h=the heat transfer coefficient, and A=the surface area for heat transfer. When convection is the dominant mode of heat transfer, as for small insects (Stevenson 1985), h is determined primarily by the size and shape of the insect and by air flow velocity in the column.

Let the time constant $\tau=Cm/hA$. Suppose that ambient temperature in the column changes linearly with time, so that $T_a(t)=a+bt$, where a is the initial temperature at time $t=0$, and b is the rate of temperature increase in the column.

Then:

$$d[T_b(t)]/dt=[a+bt-T_b(t)]/\tau$$

with initial condition $T_b(0)=a$. The solution of this linear, first-order ODE is:

$$T_b(t)=a+bt-b\tau\,[1-e^{(-t/\tau)}]$$

Recalling that $T_a(t)=a+bt$, then:

$$T_b(t)-T_a(t)=-b\tau\,[1-e^{(-t/\tau)}]$$

Note that as t approaches infinity, the temperature difference $T_b(t)-T_a(t)$ approaches $(-b\tau)$. Thus the lag of body temperature from ambient temperature is determined simply by the size, shape and mass of the insect and the air velocity in the column (determining τ) and the rate of temperature change in the column (b). For example, for *Drosophila* in our pilot experiments, $\tau=20$s; and for a temperature rise of 10°C 15 min^{-1}, $b=0{\cdot}011$°C s^{-1}. Thus the absolute maximum lag $|T_b(t)-T_a(t)|$ is small (0·22°C) but can easily be reduced by increasing the air flow rate; in our experiments the air flow rate was only 2·5 mm s^{-1}.

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