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Metabolic activity and water vapour absorption in the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae): real-time measurements by two-channel microcalorimetry

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Summary

This work describes a new calorimetric method in which the metabolic heat production and water exchange rates of an insect larva are measured simultaneously and in real time. The experimental set-up is based on two independent calorimetric cells, which are perfused by a stream of air at controlled relative humidity (RH). The resolution for metabolic heat flow and water flux is 1 μW and 5 μg h^{-1}, respectively. The method was used to investigate water vapour absorption (WVA) in drought-stressed larvae of the common mealworm *Tenebrio molitor*. It was found that during exposure to a linear increment in RH of 3% per hour, the larvae initiated WVA upon passing a threshold value of 92.7±0.6%RH. The rate of water absorption subsequently increased to reach a maximal level of 86±6 μg h^{-1}, 10–15 h after passing the threshold value. Concomitantly, the RH in the calorimetric cell was reduced to 88.6±0.5%.

The metabolic heat production of the larvae was 5–6 J h^{-1} g^{-1} wet mass in the initial part of the experiment. However, this value doubled 2–3 h prior to the onset of WVA, when the RH had reached 88%. This increase in metabolic heat production gradually tapered off over the following 24 h of WVA, during which time WVA remained high. Animals exposed to RH protocols that did not induce WVA showed no such anomalies in metabolic heat flow. This may suggest that the increased metabolism reflects the preparation of the WVA apparatus. Finally, the method was used to quantify water losses in the microgram range associated with wriggling and tracheal ventilation.

Key words: water exchange, specific corrected metabolic heat flux, controlled relative humidity, water vapour absorption threshold, mealworm, *Tenebrio molitor*.

Introduction

Several groups of terrestrial arthropods are capable of actively absorbing water from air well below its point of saturation (Dunbar and Winston, 1975; Machin and O'Donnell, 1991; Noble-Nesbitt, 1973, 1978). Active water absorption has been shown to occur in different organs including the rectum of *Tenebrio molitor* larvae, and in the two oral sacs of *Arenivaga investigata* (Machin, 1975, 1978; Noble-Nesbitt, 1970, 1973; O'Donnell and Machin, 1991). Water vapour absorption is an energy-requiring process because a substantial difference in water activity between the atmosphere and the activity of the haemolymph must be overcome (Hadley, 1994; Ramsay, 1964). Theoretical calculations and some experimental measurements have indicated, however, that the amount of energy required is relatively small compared to the total amount of stored energy resources (Hadley, 1994; Ramsay, 1964).

Since early studies by Buxton (1930) and Mellanby (1932), gravimetric methods have been most commonly used for measuring evaporative water loss in arthropods (Audsley et al., 1993; Hadley, 1994; Pelletier, 1995; Ramløv and Lee, 2000). This method assumes that mass loss and water loss are equivalent (Wharton and Richards, 1978). Changes in body mass are usually recorded at fixed intervals under known conditions of temperature and humidity (Beament, 1959; Dunbar and Winston, 1975; Edney, 1971; Machin, 1975, 1976, 1978; Machin and O'Donnell, 1991; Noble-Nesbitt, 1978; Pelletier, 1995). These measurements are often done in static systems with no air movement, which may cause water vapour gradients within the container housing the animal (Hadley, 1994). Gravimetrically determined water flux, however, is susceptible to certain errors, particularly the disruption of experimental temperatures and relative humidity (RH) during weighing (Hadley, 1994). Machin (1976) designed a flow-through system in which it was possible to simultaneously weigh the test animal and control the RH and temperature in the balance chamber, which solved the problems due to disruption when weighing the animals. More recently, flow-through respirometry has been used to measure CO_2 and water vapour production simultaneously in arthropods (Gibbs et al., 1998; Williams et al., 1998). This experimental set-up requires

temporary perfusion with dry air, which may induce a response from the experimental animal. Other investigators have developed a method to measure water loss isotopically (Wharton and Richards, 1978). However, the injection of tritiated solutions into animals introduces problems due to the puncture of the cuticle causing stress and wounds that must be treated. Water loss rates obtained isotopically are in principle unidirectional effluxes, and hence often slightly higher than the net changes determined gravimetrically (Hadley, 1994). Another possible limitation of isotope methods is the compartmentalisation of tritium in the arthropod (Hadley, 1994).

Calorimetry is used extensively for metabolic studies in animals (Dauncey, 1991; Harak et al., 1996, 1998, 1999; Kuusik et al., 1994; Lambrecht, 1998; Schmolz et al., 1999, 2002; Schmolz and Lambrecht, 2000; Schaatschmidt et al., 1995). Some of these studies use calorimetric measurements to monitor the heat flux from the animals at prefixed temperature and/or humidity. In some cases, the measurement of heat flow is supplemented by the simultaneous measurements of oxygen consumption (Harak et al., 1996, 1999). Most work in this field is performed on so-called isothermal microcalorimeters, implying instruments designed for work in the microwatt range under isothermal conditions (Guan et al., 1999; Johansson and Wadsö, 1999). In many cases, the versatility of the technique has been enhanced by the insertion of miniaturized analytical sensors into the reaction vessel, thus improving the interpretation of the results (Bäckman and Wadsö, 1991).

In this work we introduce a method combining two calorimeters, which enables the simultaneous quantification of water exchange and heat production of an experimental animal. Calorimetric approaches to vapour sorption have previously been presented by Wadsö and Wadsö (1996, 1997) and Markova and Wadsö (1999), and a methodology similar to the one employed here has previously been used for studies of the hydration of compounds of pharmaceutical interest (Lehto and Laine, 2000).

In the present study the twin calorimetric method is used to examine water exchange and related metabolic heat production in drought-stressed larvae of *T. molitor*. The results indicate that an increase in metabolism prior to the initiation of water vapour absorption reflects a sensory response to the RH of the experimental chamber.

Materials and methods

Tenebrio molitor acclimation

The larvae *Tenebrio molitor* L. were reared on flour in laboratory culture at room temperature and ambient humidity at Roskilde University. Several weeks before calorimetric measurement, larvae were placed in separate glass vials without food, light and at RH <10% over silica gel. A few larvae were acclimated with an ambient RH of approximately 30% in a flow-through system with saturated KCl-solution as water absorbent. The acclimation temperature was 21±1°C.

Instrumentation

The calorimetric measurements were conducted on a thermal activity monitor (TAM) 2277 isothermal calorimeter (Thermometric A/B, Järfälla, Sweden) equipped with an amplifier module enabling a resolution of the heat flow measurements extending into the nW range. The instrument has a total of four calorimetric channels, each of which consists of a sample cell and a reference cell mounted in a separate heat sink. This design allows independent measurement of the differential (sample – reference) heat flow in each channel, with the only restriction that the experimental temperature is the same (since all four heat sinks are in the same water bath). During measurement, the primary observable variable is simply the temperature difference between the sample and the reference; through appropriate calibration this translates into the sample-to-reference heat flow (in W). Details of a very similar, albeit less sensitive, version of this equipment have been published by its inventors (Suurkuusk and Wadsö, 1982).

In the current application, one type 2250 perfusion module (Thermometric) with a 1 ml stainless steel cell, and one type 2250 RH module (4 ml stainless steel cell), were used in each trial according to the methodology below.

The response of the *T. molitor* larvae towards changes in humidity was studied by exposing the experimental animals to a flow of atmospheric air at a controlled rate and RH. To this end, the calorimeter was equipped with a 1131 RH control module (Thermometric), which regulated two computer-controlled mass flow valves (Bronkhorst, the Netherlands). During operation, dry atmospheric air is supplied from a tank to the mass flow valves, which generate two air streams of specific flow rates. One stream was saturated with water vapour in a series of two humidifying chambers in the RH 2250

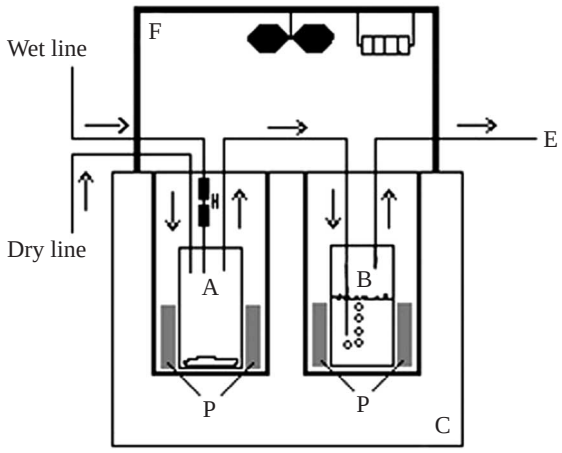

Fig. 1. A schematic drawing of the calorimetric set-up. The reference sides of the calorimetric units and heat exchangers are excluded from the drawing for clarification. A, sample cell (cell A), housing experimental animal, placed in the first calorimetric unit; B, evaporation cell (cell B), for measurements of relative humidity of the gas flow, placed in the second calorimetric unit; F, thermostated fanned box; H, humidifying chambers; P, Peltier elements; C, thermostat; E, exhaust line.

calorimetric module (H in Fig. 1), while the other was led directly to the calorimetric cell. In this way, adjustment of the rates of the two air streams allowed control of the RH in the calorimetric cell A, which houses the larvae. This control may involve keeping a constant humidity or more complicated protocols of pre-programmed linear gradients in humidity or stepwise changes.

The parts of the 2250 calorimetric modules that are outside the TAM instrument as well as the Teflon® tubing that carries the gas flow were housed in a fanned box kept at 49±0.2°C (F in Fig. 1) on top of the TAM. This prevented any measurable adsorption of vapour in the auxiliary equipment.

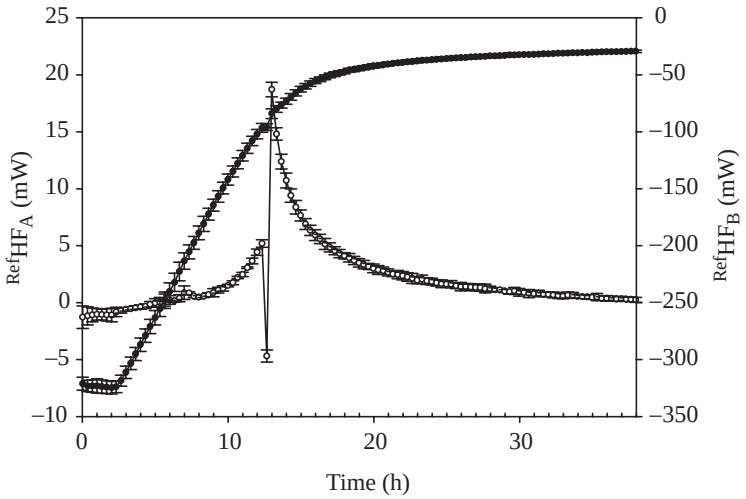

Fig. 2. Results from repeated control measurements (means ± S.D.) for cell A ($^{ref}HF_A$; open circles, N=4) and cell B ($^{ref}HF_B$; closed circles, N=6). The relative humidity was scanned from 60% to 100% over 12 h as described in detail in the text.

Methodology

The experimental approach was based on the use of two serially connected calorimetric modules, which were continuously purged with the stream of atmospheric air from the mass flow valves (Fig. 1). This set-up allowed simultaneous, real-time measurement of the metabolic activity and the water exchange of the larvae.

The air stream entered the first calorimetric cell (A in Fig. 1) at a total air flow rate J_{air} (volume/time) and a nominal RH, RH_{supply} (in %). The thermal output (or heat flow) of cell A, HF_A, signifies the combined effect of the metabolic heat and the thermal effects of water adsorbed or evaporated from the animal or the walls of the calorimetric cell. Due to these adsorption/evaporation effects, the actual RH in the calorimetric cell, RH_{cell},, which governs the water exchange of the larvae, is usually different from RH_{supply}. Subsequent to purging cell A, the air stream was led to cell B, which contained 700 µl distilled water, and served to measure RH_{cell}. Thus, saturation of the purge gas with water vapour required a small evaporation, and the associated endothermic heat flow of cell B, HF_B, directly reflected the humidity of the incoming air flow. Thus, if the vapour is considered an ideal gas:

$$RH_{cell} = 100[1 - (HF_B \times \boldsymbol{R}T) / (J_{air} \times \Delta H_{evap} \times P^*)] , \quad (1)$$

where $\boldsymbol{R}$ is the gas constant, T is the absolute temperature, ΔH_{evap} is the heat of evaporation and P* is the vapour pressure of pure water. The two latter quantities, respectively 44.0 kJ mol^{-1} (Lindstrom and Mallard, 2003) and 31.58 mbar (Weast, 1986), are taken at the experimental temperature, 25.0°C.

Along the same lines, HF_B can be used to quantify the exchange of water between the animal and the gas stream. To do so, the adsorption behaviour of cell A must be singled out in a control experiment where the reference heat flows $^{ref}HF_A$ and $^{ref}HF_B$ are measured under the same experimental conditions, but with an empty cell in calorimeter A. Once the reference heat flows are established, the difference $HF_B - {}^{ref}HF_B$ signifies whether the air stream is enriched or depleted with respect to water during its passage over the larvae in cell A, and the exchange of water, W_{H_2O} (mass/time), can be written:

$$W_{H_2O} = -(HF_B - {}^{ref}HF_B)M_{H_2O} / \Delta H_{evap} , \quad (2)$$

where M_{H_2O} is the molecular mass of water.

When the water exchange W_{H_2O} has been quantified, the calorimetric output HF_A can be analysed with respect to its constituent contributions. Hence the metabolic heat production $HF_{metabol}$ can be written:

$$HF_{metabol} = HF_A - {}^{ref}HF_A + (HF_B - {}^{ref}HF_B) , \quad (3)$$

where $^{ref}HF_A$ reflects water adsorption to the calorimetric cell and $(HF_B - {}^{ref}HF_B)$ water exchange of the animal. As it will appear below, $^{ref}HF_A$ was small, while $(HF_B - {}^{ref}HF_B)$ became significant under conditions of high humidity, where the larva absorbed significant amounts of vapour.

Mixing of gas in the volumes of calorimetric cells and the tubing introduces a delay in the detection (in cell B) of water absorbed by the animal (in cell A). This delay depends on the magnitude of the 'dead-volume' and the flow rate J_{air}. Assuming fast equilibration of humidity gradients inside cell A, the delay follows an exponential time course and can readily be quantified. In this work, however, we did not apply any explicit time correction on W_{H_2O}. Rather, we established experimentally that the effects of this delay fell to undetectable levels within about 15 min. This is a satisfactory time resolution for the current purpose.

Results

The purpose of this work is to elucidate water vapour absorption in *Tenebrio molitor* through relationships between RH_{cell}, W_{H_2O} and $HF_{metabol}$ as defined in Equations 1–3. It appears from the equations that the latter two functions rely on

a difference in heat flows of an experimental trial and a control. Hence the trial-to-trial reproducibility is essential for experimental precision. To test this, we initially measured $^{ref}HF_A$ and $^{ref}HF_B$ in a number of independent experiments with an empty A cell. Fig. 2 illustrates the average results with standard deviations indicated by the vertical bars. The experimental temperature was 25°C, J_{air} was 45 ml h^{-1}, and the time course of RH_{supply} was 2 h at 60%RH, followed by a linear increase from 60% to 100% over 12 h and constant 100% humidity for an additional 24 h. It appears that the heat flow in cell A increased over the first 15 h of experiment. This reflects the (exothermic) adsorption of water to the cell surface

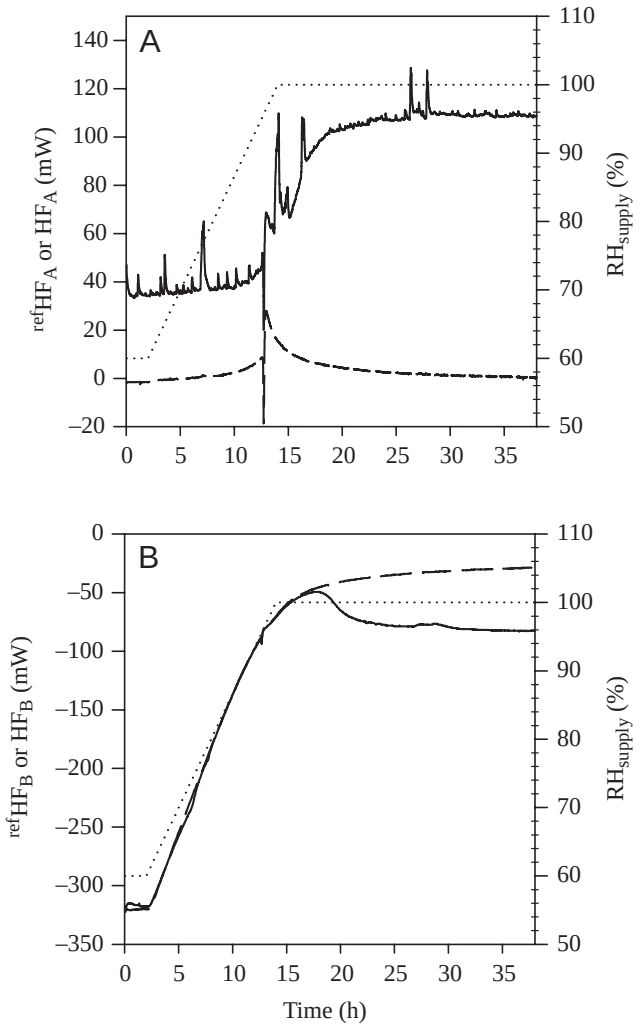

Fig. 3. Raw data for (A) cell A and (B) cell B from an experiment with a *Tenebrio molitor* larva (mass 48.26 mg; solid lines) and for a control experiment (broken lines). The dotted line shows the nominal value RH_{supply} of relative humidity of the air-flow entering cell A. In A, the heat production of the experimental animal is about 35 μW over the initial 12 h. At this point HF_A more than doubles over a time span of about 7 h. The abrupt peak in the control experiment observed after 12 h is a reproducible artefact related to the control of the flow-valves (see Fig. 2 and text). In B, a conspicuous difference between experiment (solid line) and control (broken line) is observed after 17 h. This signifies water vapour adsorption by the larva (see Fig. 4).

as the water activity in the air stream increased. As RH_{supply} approached its plateau level, $^{ref}HF_A$ decreased, and became practically zero after about 30 h, signifying that adsorption equilibrium was established. A sharp endotherm in the $^{ref}HF_A$ trace was observed after approximately 13 h. The origin of this observation is unknown, but since it was highly reproducible it was considered tenable to use the $^{ref}HF_A$ profile as shown in Fig. 2 in the calculation of $HF_{metabol}$. In any case, the numerical magnitude of this effect is rather small compared to measured HF_A values (c.f. Fig. 3). More importantly, the HF_A data in Fig. 2 show highly reproducible curves with standard deviations around 1 μW or about 1% of the heat production of a typical *T. molitor* larva, throughout the humidity scan. The data for $^{ref}HF_B$ shows the expected stepwise behaviour where the (negative) signal is proportional to (100–RH_{supply}). The rounded course at the high plateau reflects the gradual adsorption in cell A and the final value of –30 μW signifies (Equation 1) that RH_{cell} reaches a value of about 96% at the end of these trials. The standard deviation of $^{ref}HF_B$ decreased from about 5 μW at the lowest humidities to 1.5 μW at the end of the trials. These uncertainties of the control experiments translate (Equation 2) into a detection level for the measurement of water exchange of about 2–7 μg h^{-1}.

An example of raw data from a trial with experimental parameters matching those for the control experiments discussed above is shown in Fig. 3. A *T. molitor* larva with a body mass of 41.01 mg at full hydration was drought-acclimated for 48 days as described above, and transferred to the calorimeter (body mass now 33.08 mg). Fig. 3A illustrates HF_A (solid line) and RH_{supply} (dotted line). For comparison, a reference trial $^{ref}HF_A$ from Fig. 2 is also plotted (broken line) in Fig. 3A. While the heat flows and the derived RH_{cell} function (Equation 1) represent actual measurements, the values of RH_{supply} in Fig. 3 are nominal, given by the 1131 RH control unit. The accuracy of these nominal values was examined in separate experiments where cell A contained saturated salt solutions. The null-balance of heat flow when the cell contained saturated NaCl (RH=75.3% at 25°C; Clarke and Glew, 1985) suggested that at this RH the nominal values of RH_{supply} were accurate to within 0.5%RH. The heat flow HF_A in Fig. 3A shows that the larva investigated here had a heat production of about 35 μW in the first fourth of the experiment. In addition to this basic level a number of small and a few larger peaks were observed. As the humidity of the purge gas increased, HF_A increased to about three times the initial value.

Fig. 3B shows an example of raw data from cell B, HF_B (solid line). By analogy with Fig. 3A the graph in Fig. 3B is supplemented with $^{ref}HF_B$ (broken line) and RH_{supply} (dotted line). Inspection of the data shows that during the initial plateau at 60%RH, RH_B was less negative than $^{ref}HF_B$. This shows that the presence of the larva in cell A increased the humidity of the gas flow, or, in other words, that the animal was losing water. Conversely, towards the end of the trial, the observation that $HF_B < {}^{ref}HF_B$ signifies water vapour absorption in the larva.

To facilitate quantitative interpretations, the functions RH_{cell}, $HF_{metabol}$ and W_{H_2O} were calculated from raw data like

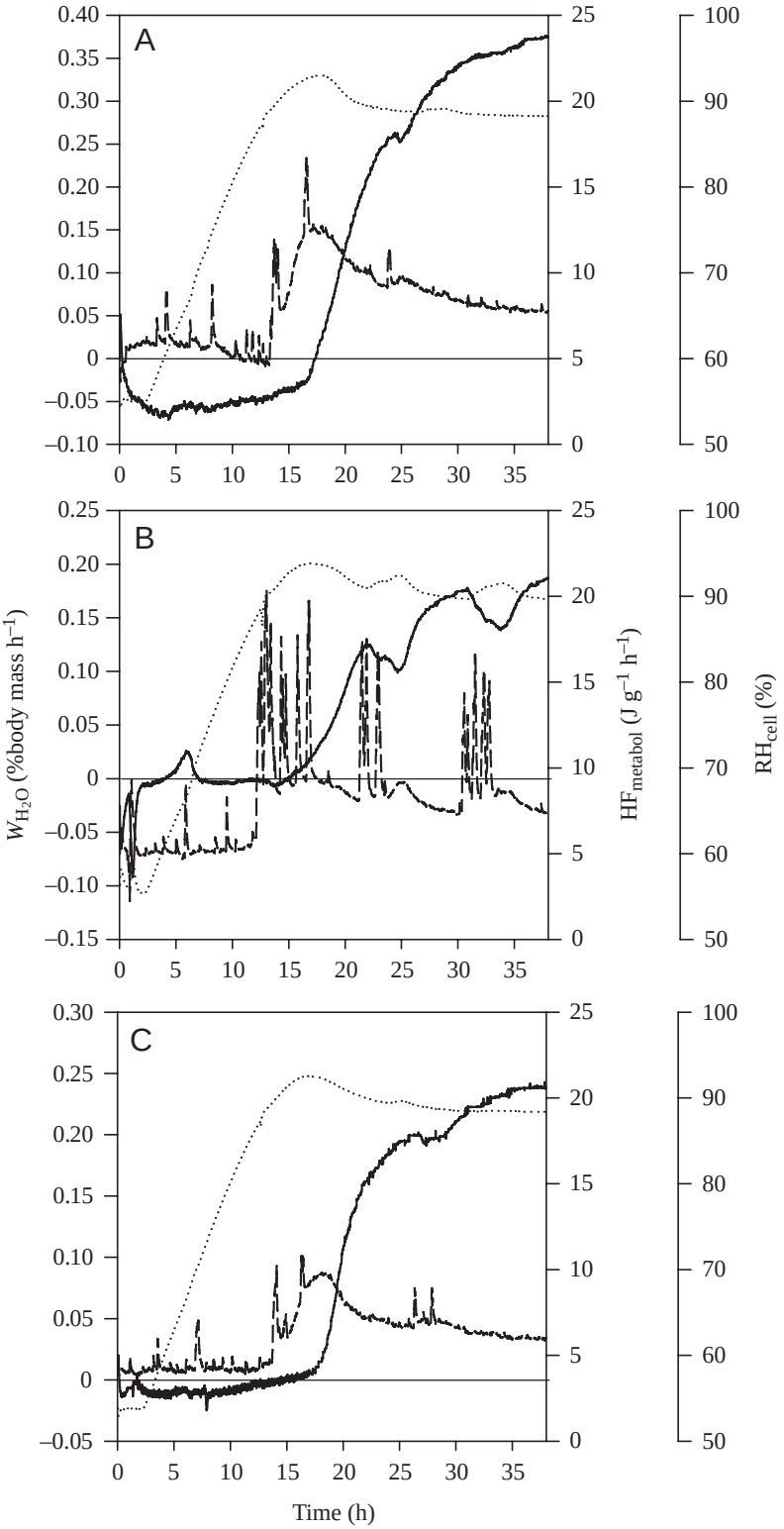

Fig. 4. Representative data showing water flux (W_{H_2O}; solid lines), corrected specific metabolic heat flow, defined in Equation 3 ($HF_{metabol}$; broken lines) and measured (see Equation 1) relative humidity (in %) of cell A (RH_{cell}; dotted line). The larvae weighed 19.79 mg (A), 45.95 mg (B) and 33.08 mg (C). For W_{H_2O} the sign is defined such that a positive value signifies net water uptake.

those in Fig. 3, and plotted against the experimental time as exemplified in Fig. 4A–C, which represents three larvae that had been pre-acclimated for 47–62 days without food. At the beginning of the calorimetric measurements the wet masses were, respectively, 19.79 mg, 45.95 mg and 33.08 mg The curves in Fig. 4 show RH_{cell}, (dotted lines), $HF_{metabol}/M_w$ (broken lines) and W_{H_2O}/M_w (solid lines), where M_w is the wet mass of the animal at the beginning of the calorimetric measurement. Since $HF_{metabol}$ and W_{H_2O} are expected to scale with the size of the experimental animal, these functions were normalized with respect to M_w before plotting the data in Fig. 4. The results in Fig. 4 and analogous data for trials not presented graphically showed several general features. Some of these are listed in Table 1, together with information of the pre-acclimation. It appears that the larvae consistently initiated WVA when RH_{cell} reached 92.7±0.6%RH and that the absorption subsequently decreased RH_{cell} to 88.6±0.5. Table 1 also lists the maximal absorption rate, which was 86±6 μg h^{-1} and independent of the body mass, and estimates of the average metabolic heat production during the initial (0–10 h) and intermediate (15–20 h) stages of the experiment.

Discussion

The current calorimetric method was devised to allow simultaneous observation of metabolic activity and water exchange during controlled changes in the ambient water vapour pressure. The critical factor for the determination of both of these functions is the precision by which HF_B–$^{ref}HF_B$ can be determined. The magnitude of this difference, which is in general much smaller than each of the heat flows, directly dictates the water exchange W_{H_2O} (Equation 2) and, under most of the conditions experienced here, it is also the most important source of error in the calculation of metabolic heat production, $HF_{metabol}$ (Equation 3). Based on the reproducibility of $^{ref}HF_B$ illustrated in Fig. 2, and similar data obtained under different experimental conditions (not shown), the uncertainty for W_{H_2O} exchange is about 2–7 μg h^{-1} (i.e. ±2–7 nl liquid water per hour), which is 1–2 orders of magnitude better than values typically obtained using gravimetric techniques (Dunbar and Winston, 1975; Machin, 1975). In addition to a satisfactory precision level, the calorimetric method may have some other advantages. It is particularly suited to elucidation of the dynamics of the processes, since the primary observable variable (heat flow) reflects a rate (e.g. water uptake) rather than an integral

Table 1. *Compilation of data from the acclimation procedure and the calorimetric measurements for larvae of* Tenebrio molitor

Flow J_{air} (ml h^{-1})	Mass (mg)		Mass loss during acclimation (%)	Start of WVA (RH_{supp})	Min. RH during WVA (RH_{cell})	W_{H_2O} max. uptake rate		$HF_{metabol}$ (J g^{-1} h^{-1})		Total uptake (mg H_2O)
	Before acclimation	After acclimation				%M_b h^{-1}	mg h^{-1}	Initial	Final	
45 (A)	24.82	19.79	20.3	92.5	88.3	0.381	7.9×10^{-2}	4.6	8.0	1.18
45 (C)	41.01	33.08	19.3	92.3	88.3	0.243	8.1×10^{-2}	4.1	6.3	1.33
45 (B)	54.90	45.95	16.3	92.7	89.5	0.189	8.7×10^{-2}	4.9	7.5	1.37
45	62.05	48.48	21.9	92.1	88.2	0.182	8.8×10^{-2}	4.9	6.5	1.12
45	94.50	84.06	11.0	92.8	88.5	0.113	9.5×10^{-2}	5.0	5.9	1.73
45	147.88	123.8	16.3	93.7	88.5	0.071	8.8×10^{-2}	2.7	3.7	1.46
100	44.47	42.62	4.2	91.3	90,1	0.263	11.2×10^{-2}	4.6	6.7	1.21
100	52.60	48.37	8.0	94.8	91.2	0.178	8.6×10^{-2}	4.9	5.8	0.32

A–C in the first column identify the data shown in Fig. 4.
WVA, water vapour absorption; RH, relative humidity; M_b, body mass (g).

quantity (e.g. body mass). As a result, the method is not prone to the uncertainty of estimating rates on the basis of small (time-dependent) changes in body mass. This aspect is illustrated in Fig. 4B and will be discussed further below. Calorimetry has previously been shown to cause limited stress to the experimental animal (Harak et al., 1998) and the current set-up is quite flexible and readily allows changes in flow rate (J_{air}), experimental temperature and gas composition.

The *Tenebrio molitor* larvae investigated produced qualitatively uniform results in the calorimetric trials, and several interesting trends can be identified. Thus, a well-defined threshold value of 92.7±0.6%RH was observed for the onset of WVA for animals exposed to a linear RH ramp increasing at a slope of 3%RH per hour (Table 1). Over the subsequent 5 h we observed a strong increase in W_{H_2O} (Fig. 4), signifying the absorption of water in the larva. The W_{H_2O} function reached a steady (maximal) value of 86±6 μg h^{-1} about 10–15 h after the threshold RH had been passed. The WVA consistently reduced RH_{cell} to 88.6±0.5% (Table 1). Interestingly, these final values of W_{H_2O} and RH_{cell} showed no dependence on the size of the experimental animal. This suggests that the WVA value observed here is limited by the magnitude of the RH gradient or the amount of water furnished by the incoming moist air. Hence, reduction of RH from 97% to 88.6% for J_{air}=45 ml h^{-1} corresponds to a water uptake of 87 μg h^{-1}. To test this further we conducted two trials, in which J_{air} was increased to 100 ml h^{-1} (Table 1). The results showed slightly elevated maximal values of W_{H_2O} (90 and 112 μg h^{-1}, respectively). The observation that the maximal uptake rate does not scale with the supply of vapour as defined by J_{air} at a fixed RH suggests that the values of W_{H_2O} recorded here are indeed close to the maximum capacity of the larvae, but a detailed analysis of this and its dependence of the body size awaits further comparative studies. The observation that *T. molitor* larvae may absorb water down to ambient activities corresponding to approximately 88%RH is in accordance with previous studies (Dunbar and Winston, 1975; Grimstone et al., 1968; Machin, 1975, 1976, 1978; Mellanby, 1932; Noble-Nesbitt, 1973, 1978; O'Donnell and Machin, 1991; Ramsay, 1964). These latter studies, however, did not report the existence of any RH threshold higher than 88%RH required to initiate WVA, although a threshold of 89.7%RH has been discussed (Coutchié and Machin, 1984). To test the existence of such a threshold more specifically, we measured the water exchange in four larvae in trials where the final value RH_{supply} was 90% (otherwise the experimental parameters were equivalent to those used in the measurements illustrated in Fig. 4). The data showed no clear increase in W_{H_2O} during the 24 h exposure to 90%RH_{supply} (corresponding to RH_{cell}=86%), confirming that the larvae investigated here require exposure to a threshold value (>90%RH) to initiate WVA.

An interesting property observed for all trials was a conspicuous increase in $HF_{metabol}$ about 13 h into the experiment (Fig. 4). At this point, the base level of heat production typically doubled (from 5 to 10 J g^{-1} h^{-1}) within a few hours. The increase in $HF_{metabol}$ consistently occurred 3 h prior to the initiation of WVA, and the heat production stayed high during the initial stages of absorption. Later, as W_{H_2O} approached a steady level, metabolic heat production gradually decreased. The experiments with a maximal RH_{supply} of 90% corresponding to a maximal RH_{cell} of about 86%, did not show WVA, and no increase in $HF_{metabol}$ was observed. Hence, we suggest that the increase in $HF_{metabol}$ and the initiation of WVA are correlated. The enhanced metabolic heat production sets in at RH_{cell}≈88%, i.e. the steady level observed during prolonged WVA, and these observations raise intriguing questions regarding a sensory regulation of WVA. For example, it seems to be of interest for future work to investigate if the enhanced metabolism reflects preparation of the WVA apparatus stimulated by a critical ambient humidity of about 88%. It could tentatively be suggested that this preparation is redistribution of ions and fluids within the cryptonephridial complex. It may also indicate a *de novo* synthesis or rearrangement of strongly hygroscopic proteins (i.e. thermal hysteresis proteins; Patterson and Duman, 1978) or other proteins earlier reported from *Tenebrio molitor* (Kroeker and

Walker, 1991). The minimal (reversible) work associated with the transport of water from RH=88% to the body of the larva is about 0.3 kJ mol^{-1} water. Using typical values of W_{H_2O} and body mass (Table 1), this translates into a heat flow of less than 0.5 J g^{-1} h^{-1}, and suggests that the enhanced metabolism directly required to sustain the maximal WVA observed here is too small to be detected in the $HF_{metabol}$ function.

The $HF_{metabol}$ traces showed a number of sharp peaks. This behaviour has been studied in detail previously, and it was concluded that the larger peaks (amplitude >20 μW) arise from wriggling movements, while smaller peaks are due to abdominal pulsations and tracheal ventilation (Harak et al., 1998; Kuusik et al., 1994). Inspection of Fig. 4B, for example, shows a clear tendency of decreased WVA during the occurrence of large peaks in $HF_{metabol}$ (wriggling) at the experimental times of 22 and 33 h. This is identified as the local minima in the W_{H_2O} function, and the effect can readily be quantified as the area of the 'dips' in W_{H_2O}. The examples in Fig. 4B suggest that each of these two periods of wriggling, which last about 3 h, is coupled to a cutback in WVA of about 60 μg water. This could be either a result of a less efficient WVA in moving larvae or (more likely) water loss through a separate process such as enhanced respiration in the active animals. A qualitatively similar observation was made for the smaller peaks (≈10 μW due to pulsation/ventilation), and analysis of the 'dips' in the W_{H_2O} function suggests a concomitant water loss of 10–15 μg over a period of about 30 min. Systematic calorimetric studies of interrelationships between respiratory activity and water loss are currently in progress.

In conclusion, we have found that calorimetry is an effective tool for real-time studies of WVA in small invertebrates. The current results show that upon exposure to a threshold value of 92–93%RH, *T. molitor* absorbs water at a rate of about 90 μg h^{-1}. During the absorption, the humidity of the experimental chamber was reduced to 88%RH despite continuous perfusion with air that was practically water saturated. The onset of WVA was preceded by a pronounced increase in the metabolic heat, and this parameter gradually decreased to the pre-WVA level over the following 24 h of water absorption. This suggests a sensatory and active control of WVA.

List of abbreviations

HF_A	thermal output of cell A
HF_B	thermal output of cell B
$HF_{metabol}$	metabolic heat production rate
J_{air}	air flow rate
$^{ref}HF_A$	thermal output for control experiment of cell A
$^{ref}HF_B$	thermal output for control experiment of cell B
RH	relative humidity
RH_{cell}	actual relative humidity in cell A
RH_{supply}	nominal relative humidity of air stream entering cell A
TAM	thermal activity monitor
W_{H_2O}	water exchange rate
WVA	water vapour absorption

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