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Phenotypic plasticity of gas exchange pattern and water loss in *Scarabaeus spretus* (Coleoptera: Scarabaeidae): deconstructing the basis for metabolic rate variation

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SUMMARY

Investigation of gas exchange patterns and modulation of metabolism provide insight into metabolic control systems and evolution in diverse terrestrial environments. Variation in metabolic rate in response to environmental conditions has been explained largely in the context of two contrasting hypotheses, namely metabolic depression in response to stressful or resource-(e.g. water) limited conditions, or elevation of metabolism at low temperatures to sustain life in extreme conditions. To deconstruct the basis for metabolic rate changes in response to temperature variation, here we undertake a full factorial study investigating the longer- and short-term effects of temperature exposure on gas exchange patterns. We examined responses of traits of gas exchange [standard metabolic rate (SMR); discontinuous gas exchange (DGE) cycle frequency; cuticular, respiratory and total water loss rate (WLR)] to elucidate the magnitude and form of plastic responses in the dung beetle, *Scarabaeus spretus*. Results showed that short- and longer-term temperature variation generally have significant effects on SMR and WLR. Overall, acclimation to increased temperature led to a decline in SMR (from 0.071±0.004 ml CO_2 h^{-1} in 15°C-acclimated beetles to 0.039±0.004 ml CO_2 h^{-1} in 25°C-acclimated beetles measured at 20°C) modulated by reduced DGE frequency (15°C acclimation: 0.554±0.027 mHz, 20°C acclimation: 0.257±0.030 mHz, 25°C acclimation: 0.208±0.027 mHz recorded at 20°C), reduced cuticular WLRs (from 1.058±0.537 mg h^{-1} in 15°C-acclimated beetles to 0.900±0.400 mg h^{-1} in 25°C-acclimated beetles measured at 20°C) and reduced total WLR (from 4.2±0.5 mg h^{-1} in 15°C-acclimated beetles to 3.1±0.5 mg h^{-1} in 25°C-acclimated beetles measured at 25°C). Respiratory WLR was reduced from 2.25±0.40 mg h^{-1} in 15°C-acclimated beetles to 1.60±0.40 mg h^{-1} in 25°C-acclimated beetles measured at 25°C, suggesting conservation of water during DGE bursts. Overall, this suggests water conservation is a priority for *S. spretus* exposed to longer-term temperature variation, rather than elevation of SMR in response to low temperature acclimation, as might be expected from a beetle living in a relatively warm, low rainfall summer region. These results are significant for understanding the evolution of gas exchange patterns and trade-offs between metabolic rate and water balance in insects and other terrestrial arthropods.

Key words: metabolic depression, respirometry, water balance, acclimatization, climate change, starvation, desiccation.

INTRODUCTION

Investigations of gas exchange patterns and the ways in which they vary in insects provide considerable insight into the dynamics and evolution of gas exchange. For example, they provide understanding of how metabolic rate variation is modulated by the gas exchange system, how the underlying control systems interact and are affected by extrinsic or intrinsic factors, and how gas exchange and metabolic rate variation evolve jointly in response to environmental conditions (Lighton, 1996; Chown and Gaston, 1999; Hetz and Bradley, 2005; Quinlan and Gibbs, 2006; Kovac et al., 2007). Consequently, much attention has been given to this topic, and particularly the way acute temperature change affects metabolic rate and gas exchange (reviewed in Chown and Nicolson, 2004; Clarke, 2003; Chown et al., 2006).

For species which show discontinuous and/or cyclic gas exchange (see Marais et al., 2005; Hetz, 2007; Contreras and Bradley, 2009; Kaiser et al., 2010), several generalities regarding the acute effects of temperature now exist. For example, in some species the pattern of gas exchange shifts from predominantly discontinuous to cyclic or continuous as trial temperature increases (e.g. Dingha et al., 2005; Kovac et al., 2007; Contreras and Bradley, 2010). Declining temperature may also induce a switch from predominantly convective to predominantly diffusive gas exchange, perhaps associated with chill coma (Lighton and Lovegrove, 1990; Kovac et al., 2007). In all species examined to date, increases in metabolic rate ($\dot{V}_{CO_2}$) with temperature are modulated by higher cycle frequencies. While in some species this is accompanied by a decline in burst volume (e.g. Buck and Keister, 1955; Lighton, 1988; Quinlan and Lighton, 1999; Vogt and Appel, 2000; Duncan and Dickman, 2001), in others burst volume remains unaltered (Davis et al., 1999; Chappell and Rogowitz, 2000; Shelton and Appel, 2001; Klok and Chown, 2005; Kovac et al., 2007) (but see Terblanche and Chown, 2010). Similar plastic responses to acute changes in other environmental conditions have also been shown in insects and other tracheated arthropods, for example, as oxygen or carbon dioxide levels are altered (Lighton and Berrigan, 1995; Chown and Holter, 2000; Hetz and Bradley, 2005; Lighton and Ottesen, 2005; Clusella-Trullas and Chown, 2008; Lighton and Turner, 2008; Terblanche et al., 2008) (for a review, see Harrison et al., 2006).

However, variation in the form of acute change comprises only one aspect of the response of gas exchange pattern and metabolic rate to environmental variation. Indeed, many comparative studies

are much more concerned with how either metabolic rate or gas exchange pattern varies among species (and sometimes populations), or how the acute response to temperature varies among them, and whether this variation should be considered a consequence of adaptive phenotypic plasticity or adaptive ecotypic variation (e.g. Sømme and Block, 1991; Ayres and Scriber, 1994; Gibbs et al., 1997; Lighton, 1998; Duncan and Byrne, 2000; Addo-Bediako et al., 2002; Irlich et al., 2009; Terblanche et al., 2009). Recent comparative work has also suggested that the way in which chronic *vs* acute responses to conditions interact might also confound interpretations of experimental studies (White et al., 2007). Despite the emphasis on the role of short- *vs* longer-term effects, and particularly the significance of plasticity, few empirical studies have investigated modulation of gas exchange characteristics from such a joint perspective. Indeed, only the work by Schimpf and colleagues (Schimpf et al., 2009) has done so, examining interactions among longer-term (acclimation) and acute variation in gas concentrations (CO_2, O_2 and water vapour) for a cockroach species, and in so doing rejecting all but the hygric hypothesis (see Chown et al., 2006) for discontinuous gas exchange (DGE). In their conclusions, Schimpf and colleagues (Schimpf et al., 2009) called for additional work examining interactions among chronic and acute exposures to particular conditions, highlighting the fact that substantial additional insight could be gained from this approach. Indeed, such insight is well demonstrated in the field of thermal biology, where acclimation effects on performance curves have long been examined [see discussion in Angilletta (Angilletta, 2009) and examples in Rako and Hoffmann (Rako and Hoffmann, 2006), Deere et al. (Deere et al., 2006) and Frazier et al. (Frazier et al., 2008)].

Here, we therefore examined the interactive effects of acute and longer-term (acclimation) temperature variation on whole-individual $\dot{V}_{CO_2}$, gas exchange pattern, and total, cuticular and respiratory water loss, using a full-factorial experimental design. We included examination of water loss characteristics because of the competing hypotheses which exist to account for variation in $\dot{V}_{CO_2}$ with acclimation and/or evolutionary responses to changing temperature. Specifically, many studies have argued that in response to low temperature environments, a range of biochemical changes (including alterations of membrane lipid composition, mitochondrial density and mitochondrial function) might result in elevated standard metabolic rate in individuals exposed to these conditions (for reviews, see Hazel, 1995; Chown and Gaston, 1999; Hulbert and Else, 2000; Pörtner, 2001; Pörtner, 2004; Clarke, 2003). By contrast, a range of work has also suggested that in response to dry conditions, insects should show lower metabolic rates to reduce the water lost *via* the respiratory system (reviewed in Chown, 2002). Much controversy surrounds both ideas, but, importantly, in comparative and laboratory acclimation studies it may prove difficult to distinguish the two ideas based on $\dot{V}_{CO_2}$ data alone, because both ideas predict low metabolic rates under higher temperature conditions (the latter hypothesis incorporating the fact that high temperatures lead to high saturation deficits) (Davis et al., 2000) [see also discussion in Chappell et al. (Chappell et al., 2009)]. By contrast, it might be predicted that if water conservation was proximally responsible for a metabolic rate response, then respiratory water loss would be equivalent among acclimation treatments, or lowest under dry conditions, whilst $\dot{V}_{CO_2}$ would show a different pattern of variation, perhaps simply in keeping with what might be expected from acute temperature change (reviewed in Keister and Buck, 1964). If, on the other hand, water conservation was not at stake, then total respiratory water loss should simply vary in concert with variation in $\dot{V}_{CO_2}$.

The work was undertaken on a dung beetle species, *Scarabeus spretus*, because the underlying form of gas exchange in the group is well known (Davis et al., 1999; Duncan and Byrne, 2000; Byrne and Duncan, 2003), and evidence exists that respiratory water loss may indeed be an important driver of variation in gas exchange pattern among species (Chown and Davis, 2003). Thus, the outcome of the present work can be interpreted in the context of the specific responses of members of this subfamily to varying conditions, rather than only by general comparison with distantly related taxa. Such specific interpretation is important because of the suggestion that variation in gas exchange characteristics may have evolved for several reasons (see Chown, 2002; Schimpf et al., 2009).

MATERIALS AND METHODS

Species collection and maintenance

Dung beetles *Scarabaeus spretus* Strassen (Coleoptera: Scarabaeidae) were collected under Cape Nature Permit no. AAA004-00077-0035 using horse manure-baited pitfall traps in the Cape Peninsula, South Africa (34°11′S, 18°24′E). Beetles were returned to the laboratory within 2 h after trap recovery whereupon they were divided into three groups at random and transferred to three 4 l plastic containers filled with clean sand from their natural habitat. Moist cotton wool and filter paper was provided in each container and containers were sprayed every other day to maintain a moist soil environment typical of their natural habitat. Beetles were fed once per week on fresh horse manure for the first 2 weeks and then deprived of food for the remainder of the respirometry recordings. The three groups were stored at 15, 20 or 25°C in climate chambers (±1.5°C, Labcon, Pretoria, South Africa) for 16 days (hereafter 'acclimation temperatures') before respirometry trials commenced (N=30 beetles per acclimation temperature).

Respirometry

Trials were undertaken at 15, 20 and 25°C for 20–24 h (hereafter 'test temperatures') on three beetles simultaneously. One beetle from each acclimation temperature was run per day in order to avoid potential confounding effects of laboratory acclimation of metabolic rate (Terblanche et al., 2004; Terblanche et al., 2007). The test temperature of each respirometry system was randomized daily to minimize any potential systematic effects among work stations. For each trial, an individual was weighed (to 0.1 mg on a Mettler Toledo AX-504 electronic balance, Columbus, OH, USA), and placed into a darkened 40 ml cuvette. Individuals were allowed to settle in the cuvettes for approximately 5 min prior to commencing the recording. Bottled air was pushed through soda lime, silica gel and Drierite columns to scrub it of residual CO_2 and H_2O. Airflow was regulated using a Side-trak (Sierra Instruments, Monterey, CA, USA) mass flow controller at a rate of 200 ml min^{-1}, over the individual in the cuvette, and into the detector cells of either a LiCor 7000 (LiCor, Lincoln, NE, USA) or LiCor 6262 carbon dioxide/H_2O analyser, that had been calibrated using a CO_2 standard and a LiCor 610 vapour pressure generator. All analysers were plumbed in differential mode. Tubing of gas analysers was kept as short as possible to improve the analyser response times. However, we also calculated response times by injecting a bolus of CO_2 into the stable analyser several times to determine the average time delay and any potential effects on DGE identification. Average response times across analysers ranged from 7 to 10 s and were unlikely to influence the determination of DGE since cycles generally lasted for >30 min (Fig. 1). The output of the analysers ($\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$) was computed/stored either *via* DATACAN V (Sable Systems, Las Vegas, NV, USA) operating in DOS in the case of the LiCor 6262,

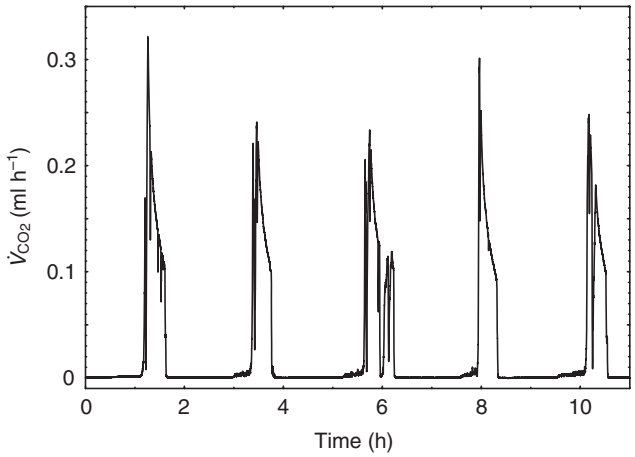

Fig. 1. Example trace of a *Scarabeus spretus* dung beetle showing discontinuous gas exchange (DGE) recorded at 15°C following 16 days of acclimation at 20°C (mass 0.7717 g). Sampling frequency 1 Hz, flow rate 200 ml min^{-1}.

or *via* LiCor software (LiCor 7000) operating in Windows XP. Data for each individual were accompanied by baseline readings for the empty cuvette, both before and after the trial. In addition, information on the activity pattern of the individual was recorded simultaneously *via* an auxiliary channel using an infrared activity detector (Sable Systems, AD-1). Following a trial, which generally lasted for 20–24 h, individuals were weighed again to check for mass loss during trials. Trials typically started at *ca*. 14:00 h–15:00 h each day and ended at *ca*. 12:00 h–13:00 h the following day.

The data for each individual were subsequently imported into EXPEDATA (Sable Systems) (*via* Microsoft Excel in the case of the LiCor 7000), baseline-drift adjusted if necessary and analysed using the customized functions of this software. First, the entire trace was analysed for the proportion of time spent active. Subsequently, the CO_2 channel was inspected for DGE patterns which only took place during resting (inactive) periods. Although individuals regularly showed periodic gas exchange, occasionally they also showed continuous gas exchange that was not associated with activity. For the DGE frequency data, only information from individuals which showed clear DGE traces was used. For standard metabolic rate data, data were taken only from resting (inactive) individuals. For resting cuticular water loss rate (WLR) data, only WLR data extracted during the closed phase of the DGE of an individual were used. Respiratory water loss was extracted from the area under the DGE burst (open phase) curve minus the cuticular water loss component area (e.g. Chown and Davis, 2003). Beetle initial body mass was used in analyses to investigate mass effects on e.g. respiratory metabolism or water loss.

Statistical analyses

Summary statistics for each of the variables were obtained using Statistica 8.0 (Statsoft, OK, USA). General linear models were initially used to investigate the extent to which variation in mean $\dot{V}_{CO_2}$ (ml h^{-1}) or DGE cycle frequency (Hz), or cuticular or total WLR (mg h^{-1}) could be explained by variation in acclimation or test temperature, mass and gas exchange characteristics [following for example Lighton (Lighton, 1991) and Davis and colleagues (Davis et al., 1999)]. Subsequently, analyses were undertaken using orthogonal polynomial contrasts which allow rank order of acclimation and test temperature to be held constant (see Huey et al., 1999).

Owing to some missing data (e.g. a beetle which remained active throughout its entire recording or individuals which never showed true DGE patterns of gas exchange) and thus marginally unbalanced sample sizes, missing data were interpolated from the mass-scaling relationships within a particular treatment group rather than sacrificing the degrees of freedom if some individuals were deleted to balance the sample sizes. This method was used to generate 3/90 of the data for WLR, burst area and resting metabolic rate and 6/90 for DGE frequency in each of the separate orthogonal polynomial contrast analyses. The proportion of time spent active during recordings was not normally distributed even after attempts to transform these data, and thus was only analysed using a generalized linear model with a log-link function and a normal distribution which is robust to violation of ANOVA assumptions (Quinn and Keogh, 2002). Because metabolic rate data were not normally distributed, and residuals of the preliminary analyses suggested violation of assumptions of general linear models, metabolic rate was log-transformed prior to final orthogonal polynomial contrast analyses. However, WLR data were normally distributed. Orthogonal polynomial contrast analyses were implemented in SAS (www.sas.com) for a three-by-three experimental study design (see Huey et al., 1999). These results did not change qualitatively when analysed using mass-specific data, suggesting that these responses were not simply a consequence of changes in body mass among treatment groups. For the sake of brevity, however, we only present results for variables that were not mass-corrected throughout.

RESULTS

Behavioural responses

Typically animals spent much less than half of the trial duration active and showed well-defined peaks of activity at dawn and dusk. Temperature had significant effects on activity in two distinct ways. First, beetles acclimated to higher temperatures had an overall reduction in time spent active during trials at all test temperatures (P<0.005). Second, a higher test temperature generally resulted in an increase in time spent active among all beetles (P=0.0486).

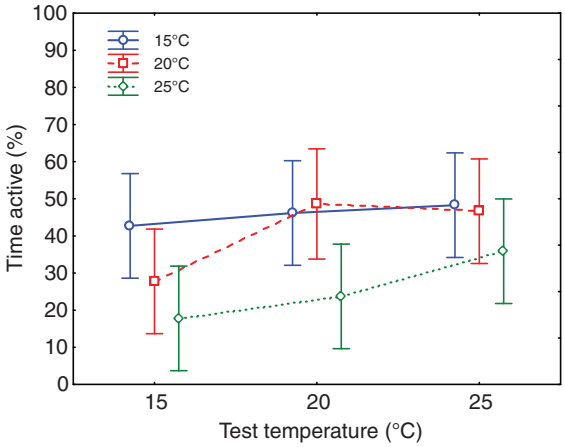

Fig. 2. Mean proportion of time spent active (%, ±95% confidence limits, CLs) during a recording (~22–24 h) for each acclimation and test temperature group. While the effect of acclimation temperature was negative and significant across the three groups (generalized linear model, log link function, normal distribution: Wald X_2=10.53, P=0.005), and the effect of test temperature was positive and significant across three temperatures (Wald X_2=6.047, P=0.0486), the interaction between acclimation and test temperature was not significant (Wald X_4=3.725, P>0.445).

However, the interaction between acclimation temperature and test temperature was non-significant (P>0.445, Fig. 2).

Body mass responses

Body mass of *S. spretus* was estimated after field collection, immediately upon arrival in the laboratory. Mean body mass did not differ among the three acclimation groups ($F_{2,25}$=0.52, P=0.601). After 16 days of acclimation, just before respirometry recordings commenced, body mass had not changed significantly in a random sample of 10 individuals per group ($F_{2,27}$=1.9459, P=0.162). After 25 days, however, body mass differed significantly, with beetles in the lowest acclimation temperature (15°C) significantly heavier than those in the mid- (20°C) and warm-temperature (25°C) groups, which did not differ from each other ($F_{2,87}$=5.4978, P=0.006; Table 1).

Metabolic rate responses

Resting (standard) metabolic rate, recorded as V_{CO_2} production, increased with test temperature, but was also negatively related to acclimation temperature (Table 1; Fig. 3). In other words, 15°C-acclimated individuals had on average higher metabolic rates at all test temperatures than 20°C- or 25°C-acclimated individuals, and across all acclimation groups test temperature had a significant positive effect. However, there was no evidence for significant interactions among test temperature and acclimation temperature (Table 2; Fig. 3). Mass-specific metabolic rate showed these same significant effects of acclimation and test temperature, but no interactions, suggesting that these responses were not simply a consequence of changes in body mass among treatment groups (Fig. 3).

DGE frequency and open phase volume

Similar to metabolic rate, DGE frequency showed a significant positive effect of test temperature and a significant negative effect of acclimation temperature (Tables 1 and 3; Fig. 4). By contrast with metabolic rate, however, the interaction between acclimation and test temperature for DGE frequency was marginally significant (Table 3). This significant interaction effect was largely a consequence of the 15°C acclimation group, in which DGE frequency was higher at test temperatures of 20°C relative to other acclimation groups, although DGE frequency at 20°C was

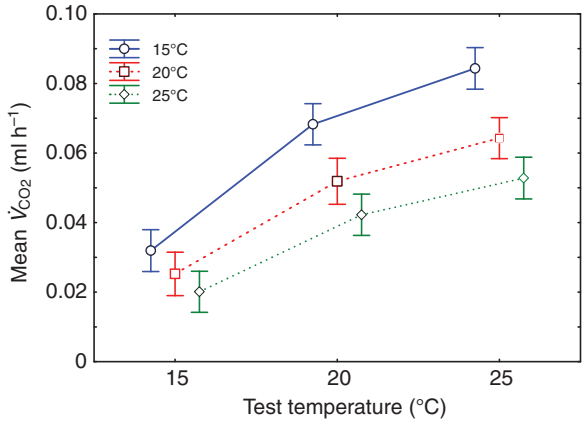

Fig. 3. The effects of acclimation and test temperature on mean resting metabolic rate (least-squares $\dot{V}_{CO_2}$ means, ±95% CLs) after correcting for body mass (ANCOVA). For results of ANOVA and ordered-factor analyses, see Table 2.

Table 1. Summary means (±s.e.m.) of body mass, resting metabolic rate, DGE frequency, and water loss, volume and duration for the closed-flutter (CF-) phase and the open (O-) phase under three acclimation and three test temperatures

					CF-phase			O-phase		
ACC (°C)	TT (°C)	Body mass (g)	Total DGE $\dot{V}_{CO_2}$ (ml h^{-1})	Total DGE frequency (mHz)	$\dot{V}_{H_2O}$ (mg h^{-1})	Volume H_2O (mg)	Duration (h)	$\dot{V}_{H_2O}$ (mg h^{-1})	Volume H_2O (mg)	Duration (h)	Volume CO_2 (ml)
15	15	0.7488±0.0249	0.037±0.004	0.219±0.027	0.791±0.458	0.601±0.361	0.82±0.10	0.959±0.576	0.476±0.396	0.48±0.03	165.1±29.2
15	20	0.7096±0.0352	0.071±0.004	0.554±0.027	1.058±0.537	0.304±0.200	0.16±0.10	1.307±0.710	0.550±0.505	0.37±0.03	184.2±56.9
15	25	0.7372±0.0465	0.089±0.004	0.556±0.027	1.950±0.624	0.393±0.147	0.26±0.10	2.261±0.626	0.676±0.262	0.27±0.03	155.8±31.9
20	15	0.6198±0.0475	0.022±0.004	0.135±0.028	0.704±0.497	0.792±0.614	1.55±0.11	0.854±0.535	0.519±0.330	0.52±0.03	165.3±61.4
20	20	0.6109±0.0477	0.049±0.004	0.257±0.030	1.074±0.458	0.689±0.454	0.70±0.11	1.283±0.545	1.043±1.137	0.49±0.03	211.8±64.7
20	25	0.6339±0.0479	0.059±0.004	0.375±0.030	1.617±0.508	0.547±0.230	0.44±0.11	1.912±0.589	0.611±0.189	0.31±0.03	155.1±41.5
25	15	0.6931±0.0681	0.022±0.004	0.133±0.027	0.723±0.348	1.162±0.804	1.79±0.10	0.850±0.423	0.545±0.317	0.61±0.03	173.2±44.8
25	20	0.5978±0.0473	0.039±0.004	0.208±0.027	0.900±0.400	0.719±0.387	0.98±0.10	1.066±0.467	1.163±2.113	0.42±0.03	185.8±61.0
25	25	0.5725±0.0524	0.048±0.004	0.318±0.027	1.432±0.205	0.470±0.284	0.56±0.10	1.606±0.246	0.577±0.291	0.32±0.03	122.5±59.8

Resting metabolic rate is given as total discontinuous gas exchange (DGE) $\dot{V}_{CO_2}$; water loss rate is given as $\dot{V}_{H_2O}$.
ACC, acclimation temperature; TT, test temperature.

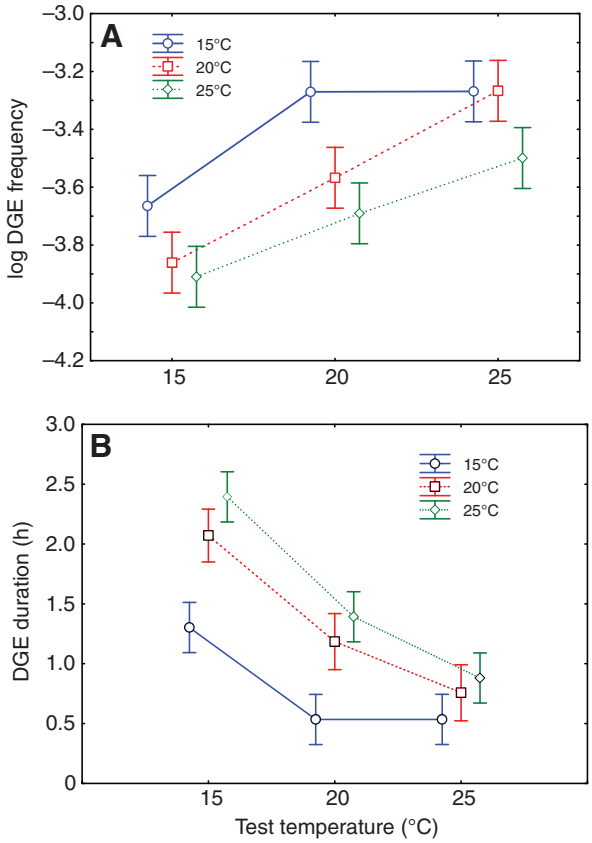

Fig. 4. The effects of acclimation and test temperature on (A) log DGE frequency (in Hz, ±95% CLs) and (B) DGE duration (in h, ±95% CLs). For results of DGE frequency analyses, see Table 3.

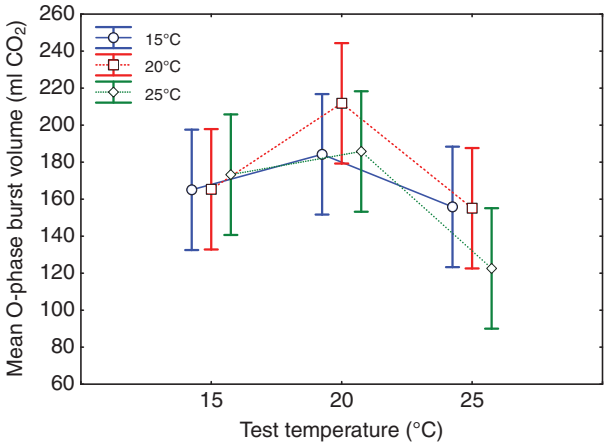

Fig. 5. The effects of acclimation and test temperature on open (O-) phase (burst) volume (in ml CO_2, ±95% CLs). For results of analyses, see Table 4.

homogeneous with that estimated at 25°C in this acclimation group (Fig. 4). Open (O-) phase (burst) volume of CO_2 was affected by test temperature but not by acclimation temperature, nor by interactions between test and acclimation temperature (Table 4; Fig. 5). Burst volume was smallest at 25°C and greatest at 20°C test temperatures (Fig. 5).

Water balance responses

Resting cuticular WLR was not affected by acclimation temperature but was positively affected by test temperature in the Type III full-factorial ANOVA (Table 5; Fig. 6). However, the single contrast analyses revealed a significant effect of acclimation temperature too, such that cuticular water loss tended to be lowest for the high acclimation temperatures, especially at a test temperature of 25°C (Table 5; Fig. 6). No significant interaction effect was detected between acclimation and test temperature for cuticular WLR (Table 5; Fig. 6). Acclimation had a significant negative effect on O-phase (burst) respiratory WLR while test temperature resulted in a significant increase in WLR (Table 6; Fig. 7A). However, acclimation did not have an effect on O-phase total water loss (Table 7, Fig. 7B). Acclimation effects on total resting WLR were non-significant while test temperature effects were highly significant in the Type III full-factorial ANOVA model (Table 8). However, in the single factor contrasts analysis, acclimation and test temperature both had significant linear effects on total WLR: negative and positive, respectively; but no interaction effects were found between acclimation and test temperature (Table 8, Fig. 8).

DISCUSSION

Acute increases in temperature resulted in an elevated metabolic rate that was mediated through an increase in DGE cycle frequency, but no significant change in O-phase burst volume. This outcome is similar to that found for five other southern African dung beetle species (Davis et al., 1999) and several other insect taxa (Chappell and Rogowitz, 2000; Shelton and Appel, 2001; Klok and Chown,

Table 2. Outputs of orthogonal polynomial contrast analyses on log resting metabolic rate (ml $CO_2\,h^{-1}$)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	**2**	**1.0689**	**0.5345**	**31.41**	**<0.0001**
TT	**2**	**2.2753**	**1.1376**	**66.85**	**<0.0001**
ACC × TT	4	0.0062	0.0016	0.09	0.9847
Contrast	d.f.	Contrast SS	MS	*F*-value	*P*
ACC linear	**1**	**1.0492**	**1.0492**	**61.65**	**<0.0001**
ACC quadratic	1	0.0198	0.0198	1.16	0.2843
TT linear	**1**	**2.1047**	**2.1047**	**123.68**	**<0.0001**
TT quadratic	**1**	**0.1706**	**0.1706**	**10.02**	**0.0022**
ACC × TT linear	2	0.0049	0.0025	0.14	0.8657
ACC × TT quadratic	2	0.0014	0.0007	0.14	0.9610
Parameter		Estimate	s.e.	*t*	*P*
ACC linear		**–0.2645**	**0.0337**	**–7.85**	**<0.0001**
ACC quadratic		0.0629	0.0583	1.08	0.2843
TT linear		**0.3746**	**0.0337**	**11.12**	**<0.0001**
TT quadratic		**–0.1847**	**0.0583**	**–3.17**	**0.0022**
ACC × TT linear		–0.0157	0.082504	–0.19	0.8493
ACC × TT quadratic		0.0068	0.14290	0.05	0.9622

Overall model R^2=0.709.
MS, mean squares; SS, sums of squares; s.e., standard error.
Bold indicates significant effects.

Table 3. Outputs of orthogonal polynomial contrast analyses on DGE cycle frequency (mHz)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	**2**	**1.3394**	**0.6697**	**23.96**	**<0.0001**
TT	**2**	**3.3646**	**1.6823**	**60.18**	**<0.0001**
ACC × TT	**4**	**0.2854**	**0.0713**	**2.55**	**0.0452**
Contrast	d.f.	Contrast SS	MS	*F*-value	*P*
ACC linear	**1**	**1.3353**	**1.3353**	**47.76**	**<0.0001**
ACC quadratic	1	0.0041	0.0041	0.15	0.7027
TT linear	**1**	**3.2697**	**3.2697**	**116.96**	**<0.0001**
TT quadratic	**1**	**0.0949**	**0.0949**	**3.39**	**0.0691**
ACC × TT linear	2	0.1218	0.0609	2.18	0.1198
ACC × TT quadratic	2	0.1636	0.0818	2.93	0.0593
Parameter		Estimate	s.e.	*t*	*P*
ACC linear		**–0.2984**	**0.0432**	**–6.91**	**<0.0001**
ACC quadratic		0.0286	0.0748	0.38	0.7027
TT linear		**0.4669**	**0.0432**	**10.81**	**<0.0001**
TT quadratic		–0.1377	0.0748	–1.84	0.0691
ACC × TT linear		0.1981	0.1057	1.87	0.0646
ACC × TT quadratic		**0.4002**	**0.1832**	**2.18**	**0.0318**

Overall model R^2=0.688.
Bold indicates significant effects.

Table 4. Outputs of orthogonal polynomial contrast analyses on O-phase burst volume (ml CO_2)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	2	4296.4	2148.2	0.80	0.4517
TT	**2**	**36697.8**	**18348.9**	**6.86**	**0.0018**
ACC × TT	4	8167.7	2014.9	0.76	0.5525
Contrast	d.f.	Contrast SS	MS	*F*-value	*P*
ACC linear	1	924.7	924.7	0.35	0.5583
ACC quadratic	1	3371.7	3371.7	1.26	0.2650
TT linear	1	8200.2	8200.2	3.06	0.0838
TT quadratic	**1**	**28497.6**	**28497.6**	**10.65**	**0.0016**
ACC × TT linear	2	5591.7	2795.9	1.04	0.3565
ACC × TT quadratic	2	2576.0	1288.0	0.48	0.6198
Parameter		Estimate	s.e.	*t*	*P*
ACC linear		–7.851	13.358	–0.59	0.5583
ACC quadratic		–25.968	23.137	–1.12	0.2650
TT linear		–23.381	13.358	–1.75	0.0838
TT quadratic		**–75.495**	**23.137**	**–3.26**	**0.0016**
ACC × TT linear		1.002	32.720	0.03	0.9756
ACC × TT quadratic		55.597	56.673	0.98	0.3295

Overall model R^2=0.185.
Bold indicates significant effects.

2005; Kovac et al., 2007), but differs from others where burst volume either declines (e.g. Buck and Keister, 1955; Lighton, 1988; Quinlan and Lighton, 1999; Vogt and Appel, 2000; Duncan and Dickman, 2001) or increases slightly (Terblanche and Chown, 2010). Longer-term variation in $\dot{V}_{CO_2}$ is similarly mediated, as indicated here by the responses to acclimation, although the higher $\dot{V}_{CO_2}$ is found at the lower acclimation temperatures. That the proximal mechanisms involved in modulating acute and longer-term responses of $\dot{V}_{CO_2}$ to temperature should be the same is perhaps unsurprising, given that a similar outcome has been found for acute and chronic exposures to gas concentrations (Schimpf et al., 2009), but has previously not been documented.

The negative relationship between $\dot{V}_{CO_2}$ and acclimation temperature is also not unexpected for insects. A wide range of studies has documented either that metabolic rate is elevated in response to low temperatures or that it is depressed in response to high temperatures (Sømme and Block, 1991; Ayres and Scriber, 1994; Chown and Gaston, 1999; Addo-Bediako et al., 2002; Terblanche et al., 2009). However, distinguishing the direction of such an effect, at least in among-species or among-population investigations, is not straightforward (Davis et al., 2000). Thus, more generally, selection might be acting either to increase metabolic rate in response to low temperatures [for various reasons, that are not without controversy (e.g. Rolfe and Brown, 1997; Clarke, 2003; Pörtner, 2004)], or might be acting to reduce metabolic rate in response to high temperatures, to improve the likelihood of survival during either starvation or desiccation (e.g. Chown, 2002; Storey and Storey, 2004; Storey and Storey, 2007). In the case of acclimation, although the existence of a change in metabolic rate with temperature is well established in several species, and has been so for some

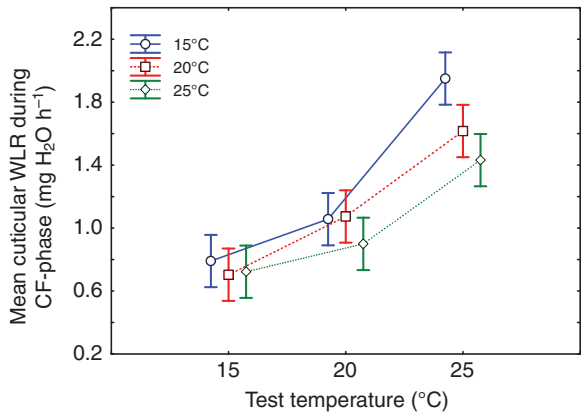

Fig. 6. The effects of acclimation and test temperature on mean cuticular water loss rate (WLR, in mg H_2O h^{-1}, ±95% CLs) during rest. CF-phase, closed-flutter phase. For results of analyses see Table 5.

time (e.g. Precht et al., 1973; Cossins and Bowler, 1987; Terblanche et al., 2005a; Terblanche et al., 2005b; Terblanche et al., 2009), the reasons for this change cannot always be unequivocally inferred, for reasons similar to those set out by Davis and colleagues (Davis et al., 2000) for interspecific comparisons. For example, we (Terblanche et al., 2009) previously suggested that in tsetse, the higher metabolic rate may be some form of acclimation response to low temperature, whereas a response to high temperature may also be likely.

One way of resolving this question is to examine directly the expected benefits of either an increase or a decline in metabolic rate. It has long been thought that a reduction in metabolic rate and a modulation of gas exchange pattern may be a means of reducing WLRs and total water loss in insects (Buck and Keister, 1955; Levy and Schneiderman, 1966; Kestler, 1985; Duncan et al., 2002). Moreover, an interspecific comparison of five dung beetle species indicated that both modulation of $\dot{V}_{CO_2}$ and duration of the periods of the DGE cycle contribute significantly to variation in respiratory WLR in the expected direction (i.e. lower metabolic rates and shorter O-phases in species with lower respiratory WLRs) (Chown and Davis, 2003). Thus, if respiratory water conservation lies at the heart of the acclimation responses found here in *S. spretus*, it might be expected that the changes in gas exchange pattern and $\dot{V}_{CO_2}$ would be associated with little change in total water loss. By contrast, if changing $\dot{V}_{CO_2}$ was a consequence of some form of metabolic response to low temperature, water loss at the various temperatures would vary in concert with the metabolic rate variation. The current results clearly demonstrate that across all treatment and acclimation temperatures, total water loss during the O-phase, the period when most water loss is likely to take place (Kestler, 1985; Lighton, 1994; Lighton, 1996) is invariant. This suggests that the modulation of $\dot{V}_{CO_2}$ taking place in this species should not be seen as an elevation in metabolic rate in response to low temperature acclimation conditions, but rather as a reduction in $\dot{V}_{CO_2}$ in response to high acclimation temperatures. Such a water conservation response is in keeping with what might be expected from a species that probably is inactive over dry summer conditions in the Mediterranean-type climate of the Western Cape Province

Table 5. Outputs of orthogonal polynomial contrast analyses on resting cuticular water loss rate (mg $H_2O\,h^{-1}$)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	2	0.9233	0.4617	2.03	0.1381
TT	**2**	**13.6331**	**6.8165**	**29.96**	**<0.0001**
ACC × TT	4	0.6830	0.1708	0.75	0.5605
Contrast	**d.f.**	**Contrast SS**	**MS**	***F*-value**	***P***
ACC linear	**1**	**0.9211**	**0.9211**	**4.05**	**0.0475**
ACC quadratic	1	0.0022	0.0022	0.01	0.9212
TT linear	**1**	**12.8908**	**12.8908**	**56.66**	**<0.0001**
TT quadratic	1	0.7423	0.7423	3.26	0.0746
ACC × TT linear	2	0.5091	0.2546	1.12	0.3316
ACC × TT quadratic	2	0.1739	0.0870	0.38	0.6836
Parameter		**Estimate**	**s.e.**	***t***	***P***
ACC linear		**–0.2478**	**0.1232**	**–2.01**	**0.0475**
ACC quadratic		0.0212	0.2133	0.10	0.9212
TT linear		**0.9270**	**0.1232**	**7.53**	**<0.0001**
TT quadratic		0.3853	0.2133	1.81	0.0746
ACC × TT linear		0.2463	0.3017	0.82	0.4167
ACC × TT quadratic		0.4539	0.5225	0.87	0.3876

Overall model R^2=0.453.
Bold indicates significant effects.

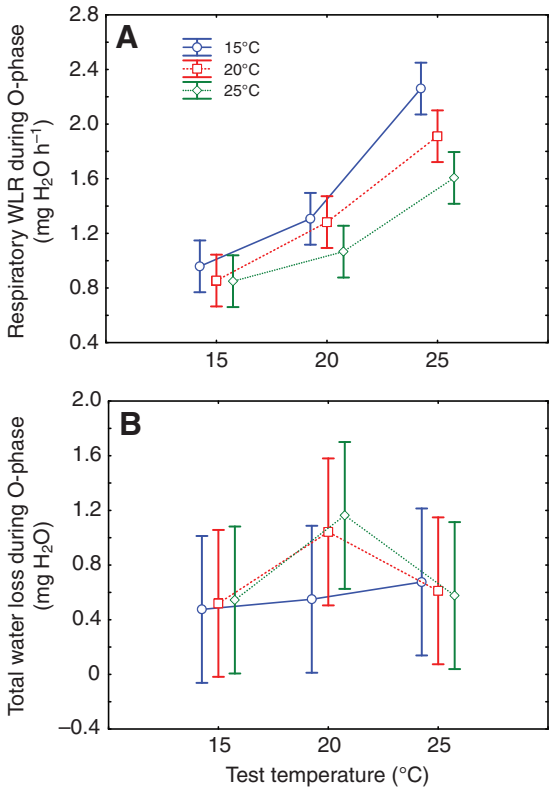

Fig. 7. The effects of acclimation and test temperature on (A) respiratory WLR (in mg $H_2O\,h^{-1}$, ±95% CLs) and (B) total respiratory water loss (in mg H_2O) during the O-phase. For results of analyses see Tables 6 and 7.

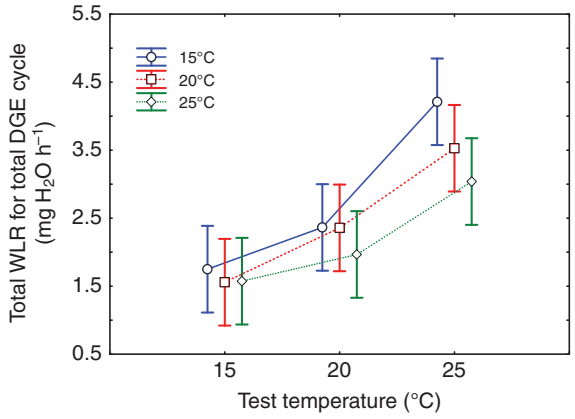

Fig. 8. The effects of acclimation and test temperature on total resting WLR (in mg $H_2O\,h^{-1}$, ±95% CLs). For results of analyses see Table 8.

Table 6. Outputs of orthogonal polynomial contrast analyses on O-phase (burst) respiratory water loss rate (mg $H_2O\,h^{-1}$)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	2	1.6798	0.8399	2.89	0.0613
TT	**2**	**16.8882**	**8.4441**	**29.06**	**<0.0001**
ACC × TT	4	0.8898	0.2225	0.77	0.5507
Contrast	d.f.	Contrast SS	MS	*F*-value	*P*
ACC linear	**1**	**1.679**	**1.679**	**5.78**	**0.0185**
ACC quadratic	1	0.001	0.001	0.01	0.9471
TT linear	**1**	**16.179**	**16.179**	**55.68**	**<0.0001**
TT quadratic	1	0.709	0.709	2.44	0.1221
ACC × TT linear	2	0.746	0.373	1.28	0.2825
ACC × TT quadratic	2	0.1435	0.0717	0.25	0.7818
Parameter		Estimate	s.e.	*t*	*P*
ACC linear		**–0.3345**	**0.1392**	**–2.40**	**0.0185**
ACC quadratic		–0.0160	0.2411	–0.07	0.9471
TT linear		**1.0385**	**0.1392**	**7.46**	**<0.0001**
TT quadratic		0.3767	0.2411	1.56	0.1221
ACC × TT linear		0.2446	0.3409	0.72	0.4752
ACC × TT quadratic		0.4049	0.5905	0.69	0.4948

Overall model R^2=0.452.
Bold indicates significant effects.

Table 7. Outputs of orthogonal polynomial contrast analyses on total water loss (mg H_2O) during the O-phase

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	2	0.6382	0.3191	0.44	0.6474
TT	2	2.6427	1.3213	1.81	0.1702
ACC × TT	4	1.5481	0.3870	0.53	0.1739
Contrast	d.f.	Contrast SS	MS	*F*-value	*P*
ACC linear	1	0.5659	0.5659	0.78	0.3812
ACC quadratic	1	0.0723	0.0723	0.10	0.7538
TT linear	1	0.1754	0.1754	0.24	0.6253
TT quadratic	1	2.4673	2.4673	3.38	0.0697
ACC × TT linear	2	0.0729	0.0365	0.05	0.9513
ACC × TT quadratic	2	1.4752	0.7376	1.01	0.3686
Parameter		Estimate	s.e.	*t*	*P*
ACC linear		0.1942	0.2206	0.88	0.3812
ACC quadratic		–0.1202	0.3821	–0.31	0.7538
TT linear		0.1081	0.2206	0.49	0.6253
TT quadratic		–0.7025	0.3821	–1.84	0.0697
ACC × TT linear		0.1086	0.5404	0.20	0.8413
ACC × TT quadratic		1.0061	0.9360	1.08	0.2856

Overall model R^2=0.076.

of South Africa. In these environments rainfall is typically low in the hot summer months and where adults probably survive the summer (Davis, 1993; Davis, 1996; Davis, 1997). Furthermore, this conservation response is also observed in animals exposed to stressful conditions and would probably also ensure a reduction of maintenance metabolic costs (Hoffmann and Parsons, 1997; Clarke, 1993), even if the metabolic downregulation is not equivalent in magnitude to that typical of aestivation (Storey and Storey, 2004; Storey and Storey, 2007).

If the conservation of water by modulation of respiratory water loss does account for the modulation in $\dot{V}_{CO_2}$ and gas exchange pattern found during acclimation, similar responses should be found for cuticular water loss. Cuticular WLRs are typically much higher than those found for respiratory water loss (Hadley, 1994; Lighton, 1998; Chown, 2002), and it would make little sense from the perspective of water conservation for a response to be effected for respiratory water loss if cuticular WLRs remained unchanged or increased with an increase in acclimation temperature. Our results revealed that WLRs are reduced in response to high temperature acclimation both for cuticular and respiratory water loss, resulting in a substantial overall decline in WLR in high temperature-acclimated animals especially at the highest test temperature (see Figs 6–8). Thus, the modulation of $\dot{V}_{CO_2}$ and gas exchange pattern as a water conservation response is at least not contradicted by the cuticular or overall WLR response, providing additional circumstantial evidence for our findings.

That cuticular WLRs should be partially reduced in response to high temperature acclimation is perhaps an unusual finding. Only a handful of studies have investigated changes in WLR in arthropods in response to thermal acclimation, with mixed responses. Several of the studies have demonstrated a decline in WLR at higher temperatures as we found here (e.g. Hadley, 1977; Toolson, 1982; Toolson and Kuper-Simbron, 1998; Chown et al., 2007; Leinaas et al., 2009), whereas others have found no effect (e.g. Gibbs et al., 1998; Terblanche et al., 2005b). In this particular instance, the response was particularly pronounced at the highest test temperature, suggesting that the WLR acclimation response could well be considered a beneficial one [see p. 1917 of Leroi et al. (Leroi et al., 1994)]. The orthogonal polynomial contrast analyses for cuticular, respiratory and total WLRs certainly bear out this idea [see discussion in Huey et al. (Huey et al., 1999)]. Whilst a strictly *a priori* hypothesis-testing approach is typically recommended for investigations of the beneficial acclimation hypothesis, it was not readily clear what the expectations might have been for water loss in the ways that these ideas have previously been framed (e.g. Huey et al., 1999; Deere and Chown, 2006). Nonetheless, had the orthogonal polynomial contrast analyses not revealed a decline in WLRs with acclimation temperature, the beneficial acclimation hypothesis would obviously have had to be rejected. Moreover, it has also been suggested that investigations of phenotypic plasticity and the extent to which it might be considered adaptive should move away from the hypothesis categorizations that have, to date, typically been used in the field (Angilletta, 2009).

By comparison with the other dung beetle species investigated in the region, *S. spretus* has a $\dot{V}_{CO_2}$ comparable to that of similar-sized *S. striatum* (Davis et al., 1999). Relative to the inter-specific metabolic rate mass-scaling relationship for insects in general, *S. spretus* metabolic rate was within the range of metabolic rates from the

compiled data presented in Chown et al. (Chown et al., 2007) for an insect of its body mass, albeit slightly lower than predicted from the equation alone (3.061 logμW predicted *vs* 2.510 logμW recorded). However, this 'overestimation' by the consensus equation can be attributed to the conversion between log and antilog scales which is a common issue when switching between prediction and line-fitting applications of allometric equations (Hayes and Shonkwiler, 2006; Hui et al., 2010), and the fact that body mass changes readily in response to rearing temperature (see Table 1 and Results). Relative cuticular and respiratory WLRs are well within the range of other species found to date (e.g. Chown and Davis, 2003). Whilst the latter are frequently reported they are likely to be of limited use in understanding the adaptive responses of insects to changing environmental water availability because both cuticular and respiratory water loss might be modulated simultaneously (Chown, 2002; Chown and Davis, 2003). The current study shows that this would also be the case for investigations of acclimation responses, or investigations of phenotypic plasticity of water loss more generally. Thus, we recommend that the convention of placing any weight on the interpretation of proportional contributions of these two avenues of water loss largely be abandoned in favour of reporting the empirical values for both routes. Empirical water loss values could easily be converted to proportions if required, but providing the former would more easily enable comparative analyses of the kind that have been undertaken for WLR and metabolic rate more generally (e.g. Addo-Bediako et al., 2001; Addo-Bediako et al., 2002).

Table 8. Outputs of orthogonal polynomial contrast analyses on total resting water loss rates (mg $H_2O\,h^{-1}$)

Source	d.f.	Type III SS	MS	*F*-value	*P*
ACC	2	5.087	2.543	2.48	0.0898
TT	**2**	**60.856**	**30.428**	**29.71**	**<0.0001**
ACC × TT	4	3.112	0.778	0.76	0.5546
Contrast	**d.f.**	**Contrast SS**	**MS**	***F*-value**	***P***
ACC linear	**1**	**5.086**	**5.086**	**4.97**	**0.0286**
ACC quadratic	1	0.001	0.001	0.00	0.9910
TT linear	**1**	**57.953**	**57.953**	**56.59**	**<0.0001**
TT quadratic	1	2.903	2.903	2.83	0.0961
ACC × TT linear	2	2.480	1.240	1.21	0.3032
ACC × TT quadratic	2	0.6316	0.3158	0.31	0.7355
Parameter		**Estimate**	**s.e.**	***t***	***P***
ACC linear		**–0.5823**	**0.2613**	**–2.23**	**0.0286**
ACC quadratic		0.0051	0.4526	0.01	0.9910
TT linear		**1.9656**	**0.2613**	**7.52**	**<0.0001**
TT quadratic		0.7620	0.4526	1.68	0.0961
ACC × TT linear		0.4908	0.6400	0.77	0.4454
ACC × TT quadratic		0.8588	1.1086	0.77	0.4408

Overall model R^2=0.454.
Bold indicates significant effects.

In conclusion, our study has demonstrated that acclimation effects on $\dot{V}_{CO_2}$ and gas exchange characteristics are more likely to be a beneficial acclimation response to effect water conservation under high temperatures than one to elevate metabolic rate under cool conditions. Such an outcome is in keeping with what is generally known about ectotherm responses to stressful conditions (Storey and Storey, 2004; Storey and Storey, 2007). Nonetheless, this does not necessarily mean that some form of low temperature metabolic response is unlikely to be characteristic of insects or other organisms generally [see Pörtner (Pörtner, 2004) for rationale], but rather that it is unlikely in southern African dung beetles. Moreover, by measuring WLR, the conundrum posed by Davis and colleagues (Davis et al., 2000) of attributing the change in $\dot{V}_{CO_2}$ to an appropriate proximal mechanism can be overcome. In consequence, we recommend that future studies measure both $\dot{V}_{CO_2}$ and $\dot{V}_{H2O}$ as a matter of course when investigating the reasons for metabolic rate variation among treatments, populations or species of insects, especially if the idea of metabolic cold responses are being tested, and that metabolic depression in response to high temperature, to avoid either starvation or desiccation, always be included as an alternative hypothesis.

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