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Metabolic rate does not decrease with starvation in *Gryllus bimaculatus* when changing fuel use is taken into account

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Abstract. Many behavioural traits are considered to be condition-dependent, reflecting the differential allocation of resources to fitness-related traits and maintenance, although the physiological underpinnings of condition dependence are not well understood. In the present study, the hypothesis that condition dependence in male *Gryllus bimaculatus* De Geer is mediated by a decrease in metabolic rate with declining condition is tested. CO_2 production is measured by flow-through respirometry, with insect condition manipulated through starvation. Crickets starved for 7 days have lower CO_2 emission rates than individuals starved for only 24 h. However, carbohydrate reserves are depleted in the first 3 days, suggesting that the initial metabolism is primarily fuelled by carbohydrate, with a shift to lipid stores after 3 days. If the metabolic rate is estimated using respiratory quotients reflecting this shift in fuels, there is no difference in metabolic rate between crickets starved for 24 h and 7 days, suggesting that metabolic rate does not decrease with declining condition. This implies that a decrease in metabolic rate during starvation may not be a general pattern in insects, and emphasizes the need to consider fuel use during metabolic rate estimation in starvation studies.

Key words. Energy reserves, Gryllidae, orthoptera, Respiratory exchange ratio, Respiratory Quotient, starvation.

Introduction

The concept of condition is applied in many contexts in behavioural ecology and is central to the study of sexual selection (Tomkins *et al*., 2004). Condition is seen as a meta-trait that depends on an individual's genetic quality, and therefore influences fitness (Rowe & Houle, 1996; Kotiaho *et al*., 2001; Hunt *et al*., 2004; Tomkins *et al*., 2010). In behavioural ecology studies, condition is usually estimated by proxy; for example, body size (David *et al*., 1998; Wilkinson & Taper, 1999; Bjorksten *et al*., 2000), asymmetry (Møller & Swaddle, 1997) and energy reserves (Milinski & Bakker, 1990; Otronen, 1995). The assumption behind these estimates is that they reflect an organism's integrated ability to respond to the environment and to assimilate energy, and that they have the potential to capture the sum total of variation among individuals (Blanckenhorn & Hosken, 2003). In laboratory studies, the manipulation of phenotypic condition is typically achieved through diet (David *et al*., 1998; Kotiaho, 2000). For example, individuals are food-restricted to generate low condition or supplied with *ad libitum* food to achieve good condition (Bjorksten *et al*., 2000; Kotiaho, 2000). However, there are few experimental systems in which the physiological responses to condition-manipulation can be linked directly to fitness traits (Lailvaux & Irschick, 2006).

Many insects are reported to reduce their metabolic rate in response to decreased energy availability during periods

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of reduced food ability, such as dry summers or cold winters, in which diapause is prevalent (Chown & Nicolson, 2004). Feeding results in a temporary increase in metabolic rate associated with the costs of processing and digestion (specific dynamic action), and nonfeeding insects thus have lower metabolic rates than those that are feeding (Bennett *et al*., 1999; Bradley *et al*., 2003). Beyond this specific dynamic action, starvation is reported to result in a reduction of metabolic rate in insects, particularly *Drosophila*. Djawdan *et al*. (1997) show that all populations of *Drosophila melanogaster* selected for desiccation or starvation resistance (as well as controls) have reduced CO_2 emission rates when starved, indicating that, at least in the case of *Drosophila*, the metabolic rate appears to be plastic in response to the energy reserves of the insect, even in the absence of a strict diapause; for definitions of diapause, see Kostal (2006). However, starvation can also bring about changes in fuel use (e.g. switching from primarily carbohydrate metabolism to primarily lipid metabolism; Chown & Nicolson, 2004; but see also Marron *et al*., 2003). Changes in fuel use alter the respiratory exchange ratio (or respiratory quotient, RQ); thus, it is important to consider respiratory substrate when comparing metabolic rate estimated from raw oxygen consumption or CO_2 production (Lighton, 2008; McCue, 2010); it is generally assumed that starved insects metabolize lipid reserves (Chown & Nicolson, 2004), although *Drosophila* appear to utilize primarily a mix of fuels when starved (Marron *et al*., 2003).

Crickets are widely used in studies of the condition dependence of behavioural traits (Holzer *et al*., 2003; Hunt *et al*., 2004), primarily because, in most species, males sing to attract females. Singing in crickets is known to be metabolically expensive (Hack, 1998) and there is evidence that song production in crickets is condition-dependent, with larger males being able to produce higher chirp and pulse rates that are preferred by females (Simmons, 1988). In a wild population of *Gryllus campestris*, the amount of singing activity of smaller males is associated with the number of offspring that they leave in the next generation (Rodríguez-Muñoz *et al*., 2010). In the present study, *Gryllus bimaculatus* De Geer is used. In *G. bimaculatus*, females are known to mate with several males in the laboratory (Tregenza & Wedell, 1998) and in the wild (Simmons, 1988; Bretman & Tregenza, 2005). *Gryllus bimaculatus* has become a laboratory model system for studies of sexual selection because song traits are a large component of female choice, as used in the present study. The clear evidence for strong sexual selection and a central role for metabolically expensive singing in this species indicates that energy availability and partitioning are expected to be strongly linked to performance.

To understand the mechanisms of resource partitioning, it is necessary to demonstrate a link between condition and the consumption of resources. In the present study, *G. bimaculatus* is used to test the hypothesis that the manipulation of condition (starvation) will lead to conservation of resource use (i.e. a decrease in metabolic rate). Metabolic rate is measured and it is predicted that crickets with reduced access to resources (starved) will depress their metabolic rate relative to those with adequate resources and body stores.

Materials and methods

Animals

Gryllus bimaculatus were derived from animals caught at La Pobla deVallbona near Valencia, Spain in 2004 (approximately ten generations in the laboratory). Stocks were maintained in 1-L boxes with excess food (rodent diet) and water under an LD 16 : 8 h photocycle at 28 °C. Late-instar nymphs were separated as soon as they could be sexed. Adults were removed daily and housed individually. Males were randomly assigned to one of two feeding regimes: starved for 7 days (7-day starved treatment), and food for 6 days and starved for 1 day (1-day starved treatment). All crickets had continuous access to water during starvation. All measurements were taken at 7 days post-adult eclosion.

Respirometry

Standard metabolic rate was estimated by CO_2 emission, measured using flow-through respirometry. All measures were made sequentially on seven individual crickets per run between 09.00 and 16.00 h when 7-day-old crickets are inactive (Tomioka & Chiba, 1982), under weak fluorescent lighting (Arcadia 18 W plant growth lamp; Arcadia, U.K.). The order of crickets was randomized to avoid any effects of time spent in the chamber. Briefly, a LI-7000 infra-red gas analyzer (Li-Cor, Lincoln, Nebraska) was attached to a Sable Systems (Las Vegas, Nevada) RM8 eight-channel multiplexer housed inside an MIR 553 incubator (Sanyo, Japan), which was set at 23 °C. Compressed zero air (a 21% O_2, 79% N_2 mix) was scrubbed of any residual CO_2 and water (using ascarite and drierite respectively) and fed through the multiplexer and into 125-mL cuvettes housing the crickets at 120 mL min^{-1} (controlled at ± 1% with a mass-flow control metre; Sierra Instruments, Monterey, California). The multiplexer regulates the flow to individual crickets, and one empty channel was used as a blank for initial and final baseline readings to control for drift. The mass-flow metre regulated airflow through each chamber for 1 h at a time, with sampling every second. Metabolism of the seven crickets was measured sequentially for 1 h each. A thermistor probe monitored temperature next to the cuvettes in the incubator. CO_2 emission and temperature data were acquired at 1 Hz via a Sable Systems UI2 analog-digital interface connected to a PC running Sable Systems EXPEDATA software. Crickets were weighed (±0.01 g with a Sartorius balance; Sartorius, Germany) before respirometry. After CO_2 measurement, crickets were killed by freezing at −20 °C and used for either lipid or carbohydrate content assays.

Extraction of metabolic rate data was conducted using EXPEDATA. Only CO_2 emission data from the final 15 min of recording were used to calculate the metabolic rate. Even if complete washout did not occur, this would not change the conclusions. Activity of the animals was clearly visible in CO_2 traces and these data were not used to calculate the standard metabolic rate. The most stable portion of the recording trace (minimum of 300 readings) was chosen to calculate the mean CO_2

emission rate over at least 5 min. Because the crickets sometimes showed cyclic gas exchange, the data to be analyzed included two to four complete cycles.

CO_2 production was converted to metabolic rate (MR, in W) using the equation:

$$MR = (15.97 + (5.164 \times RQ)) \times (VCO_2/RQ) \quad (1)$$

according to Lighton (1991), using an RQ of 0.84 (assuming a fuel mix of approximately 70% carbohydrates and some lipids) and 0.71 (assuming primarily lipid metabolism; Withers, 1992), with VCO_2 in mL min^{-1}. It was assumed that 1-day starved crickets would still have useable carbohydrate stores, whereas 7-day starved crickets would be using primarily lipids, so metabolic rates were compared among the treatments assuming an RQ of 0.71 for 7-day starved animals, and 0.84 for 1-day starved animals, using an analysis of covariance (ANCOVA) with fresh mass as a covariate in PROC GLM in SAS, version 9.2 (SAS Institute, Cary, North Carolina).

Lipid content

Lipid contents were calculated using chloroform extraction modified from Sinclair & Chown (2005). Lipid contents were calculated for $n = 13$ individuals from 1-day and $n = 15$ individuals from 7-day starved treatments. The abdomens of dead, frozen crickets were punctured four times with a sharp probe. They were weighed again (wet mass) and dried in an oven at 40 °C for 24 h (dry mass). Lipids were then extracted in three changes of chloroform–methanol mix (24 h each) before being dried overnight to obtain lipid-free dry mass. Lipid and water contents were compared among treatments using an ANCOVA with dry mass as a covariate using PROC GLM in SAS.

Carbohydrate content

Total carbohydrate content was assessed by colourimetric assay (Gefen *et al*., 2006). Whole crickets ($n = 5$, 1-day, $n = 7$, 7-day) of known mass were ground in 5 mL of 0.05% Tween 20 (Sigma-Aldrich, U.K.) using a motor homogenizer and 500 μL of the supernatant was removed for testing. Ten microlitres of the enzyme *Rhizophus* amyloglucodase (Sigma-Aldrich) was added to 10 μL of sample or 10 μL of glycogen standard (oyster type II glycogen; Sigma-Aldrich) and left to stand at room temperature overnight. The next morning, 90 μL of liquid glucose reagent (Alpha Laboratories, U.K.) was added and the absorbance measured at 340 nm in a Spectramax 384plus (Molecular Devices, U.K.). Sample values were compared with standard curves of known amounts of glucose. Carbohydrate content was also determined in an additional sample of 7-day-old crickets that had been starved for 3 days before measurement. Carbohydrate content was expressed as mg glucose equivalents and compared among treatments using ANCOVA with fresh mass as a covariate using PROC GLM in SAS.

Movement

To assess whether differences in metabolic rate could be attributed to a difference in activity levels, a subsample of crickets from each feeding treatment were observed under the same conditions that were employed for measuring the metabolic rate. Crickets were placed inside respirometry cuvettes and allowed to acclimatize for 5 min. Activity was observed once every 30 min for 6 h, scoring 0 for no movement and 1 for movement. Movement was compared among treatments using a rank-sum test.

Unless otherwise stated, data are reported as the mean ± SE.

Results

Crickets starved for 7 days had significantly lower mass and carbohydrate content than crickets starved for only 1 day, although water and lipid content (which were lower) did not differ significantly between the treatments after analysis of covariance. This suggests that the carbohydrate stores were depleted between days 1 and 7 (Table 1). Crickets starved for 3 days had a carbohydrate content of 2.88 ± 0.14 mg $g^{-0.63}$, which was lower than that of 1-day crickets, although this did

Table 1. Body composition and metabolic rate (MR) of adult male *Gryllus bimaculatus* crickets starved for 1 or 7 days.

	1-day starved	7-days starved	Statistics
Mass (g)	0.64 ± 0.04 (16)	0.53 ± 0.01* (45)	$t_{51} = 3.3$, $P = 0.002$
Water content (g)	0.393 ± 0.027 (13)	0.297 ± 0.018 (15)	$F_{1,25} = 2.33$, $P = 0.139$
Lipid (g)	0.039 ± 0.006 (13)	0.015 ± 0.003 (15)	$F_{1,25} < 0.001$, $P = 0.976$
Mass-specific lipid (g $g^{-0.63}$)	0.057 ± 0.008	0.026 ± 0.005	—
Carbohydrate (mg $g^{-0.63}$)	3.32 ± 0.16 (13)	2.57 ± 0.14* (18)	$F_{2,15} = 3.82$, $P = 0.046$
CO_2 production (μL min^{-1})	1.660 ± 0.052 (16)	1.215 ± 0.060* (37)	$F_{1,50} = 9.84$, $P = 0.003$
MR (μ W $g^{-0.63}$, assuming using lipids; RQ = 0.71)	1029.1 ± 29.9	853.7 ± 39.1	—
MR (μ W $g^{-0.63}$, assuming using mix of lipids and carbohydrates; RQ = 0.84)	899.5 ± 26.2	746.2 ± 34.2	—

A scaling exponent of 0.63 was taken from the observed mass-metabolic rate relationship, and used to calculate mass-specific values (although analyses were performed using an analysis of covariance on raw data with mass as a covariate). Comparison of metabolic rates must account for different RQ at 1 and 7 days; for statistical comparison, see text. An asterisk indicates a significant difference between days 1 and 7 for comparisons of everything, except MR. Numbers in parentheses are sample sizes. Data are reported as the mean ± SE.

not differ significantly from those starved for 7 days ($F_{2,15} = 3.82$, $P = 0.046$; Tukey's test 1 versus 3: $P = 0.042$, 3 versus 7: $P = 0.268$). Thus, it appears that crickets starved for 1 day are metabolizing carbohydrates, whereas crickets starved for 7 days have only lipid stores remaining. Therefore, metabolic rates were calculated from CO_2 emission rate using an RQ of 0.84 (assuming a mix of carbohydrate and lipids as fuel) for 1 day and 0.71 (assuming almost entirely lipids) for 7-day individuals.

Crickets showed generally cyclical patterns of gas exchange without completely closed phases, although there was no apparent difference in gas exchange pattern between the treatments (Fig. 1). Absolute CO_2 emission rate decreased with starvation ($F_{1,50} = 9.84$, $P = 0.003$ and both CO_2 emission and metabolic rates were significantly related to body mass (CO_2: $F_{1,50} = 12.27$, $P = 0.001$; metabolic rate: $F_{1,50} = 11.33$, $P = 0.002$). However, when CO_2 emission rate was converted to metabolic rate to account for the different energy reserves of the treatments, starvation did not significantly change metabolic rate ($F_{1,50} = 1.1$, $P = 0.300$). A linear regression of $\log_{10}$-transformed metabolic rate on mass gives a scaling exponent of 0.628 (Fig. 2).

Crickets were rarely seen to move in the chambers (in 732 observations movement was detected on only 29 occasions) and rank-sum scores did not differ significantly between days 1 and 7 ($z = 0.47$; $P = 0.32$).

Discussion

Standard metabolic rates after 1 and 7 days of starvation show no condition-dependence in *G. bimaculatus*. Lipid contents are not significantly reduced after 7 days, although carbohydrate contents decline from 1 to 3 days, and do not differ between crickets starved for 3 and 7 days. Thus, it appears that crickets initially use a mix of carbohydrate and lipid metabolism (and that the mass lost is primarily carbohydrate) but, after the carbohydrate stores are depleted, lipids are primarily utilized.

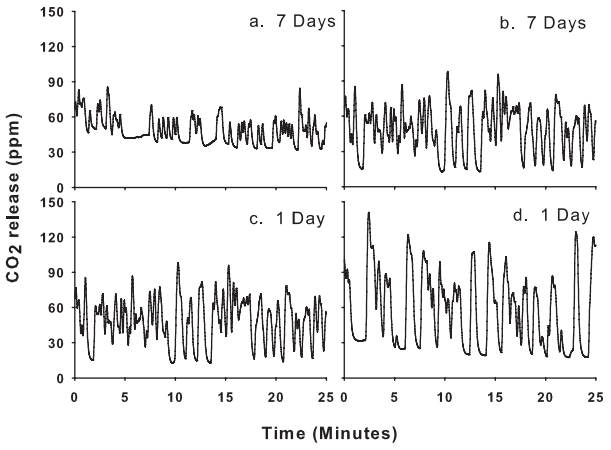

Fig. 1. Example traces of CO_2 emission by *Gryllus bimaculatus* crickets starved for 7 (a, b) and 1 (c, d) days. Masses of the crickets were 0.64, 0.47, 0.69 and 0.60 g for a, b, c and d, respectively.

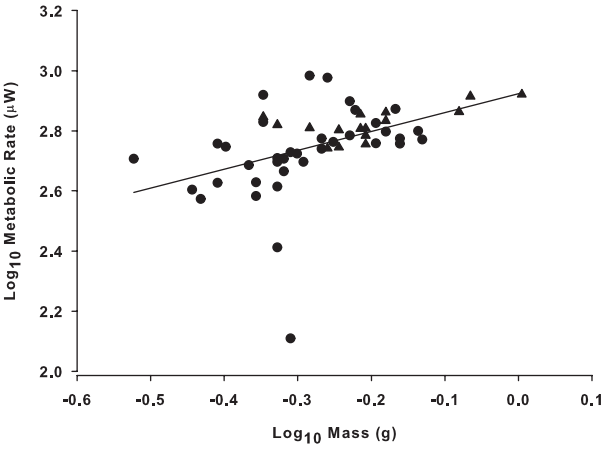

Fig. 2. Relationship between mass and metabolic rate in *Gryllus bimaculatus* crickets. Metabolic rates were expressed in μW, calculated from raw CO_2 emission rate using a respiratory quotient of 0.84 for day 1 (triangles) and 0.71 for day 7 crickets (circles). Because the metabolic rates of these two groups do not differ, the data were pooled for the least-squares linear regression, represented by the solid line. Regression equation: metabolic rate $= 2.923 \times \text{mass}^{0.63}$; $r^2 = 0.207$.

Drosophila also use a mix of fuels during starvation (Marron *et al*., 2003), consistent with other insect species (Chown & Nicolson, 2004). Metabolic rates, assuming differing RQ for crickets starved for 1 and 7 days, do not differ between starved and recently fed crickets, and are slightly lower than those measured in male *G. lineaticeps* at 25 °C (approximately 2200 μW $g^{-0.75}$ assuming an RQ of 0.84, a scaling exponent of 0.75; Hoback & Wagner, 1997). If the carbohydrate is assumed to be the only fuel in the 1-day crickets, this would further decrease the calculated metabolic rate towards the 7-day value that is based on the assumption of lipid utilization, so the conclusion presented here is conservative. The intraspecific scaling exponent for metabolic rate is 0.63, which is somewhat below the consensus value of 0.82 among insect species but within range of other values from intraspecific studies (Chown *et al*., 2007).

Although absolute and mass-specific CO_2 emission rate appear to indicate a decrease in metabolic rate in starved crickets, our assumptions about RQ in these crickets indicate that metabolic rate may not actually decrease, and that the observed difference is a consequence of a shift in fuel use. This is consistent with direct calorimetric observations of *D. melanogaster* metabolic rate during caloric restriction (Hulbert *et al*., 2004), and contrasts with respirometric studies in *D. melanogaster* (Djawdan *et al*., 1997), tsetse flies (*Glossina morsitans centralis*) (Terblanche & Chown, 2007) and several other insect taxa (e.g. Lepidoptera; Bennett *et al*., 1999; Coleoptera: Renault *et al*., 2003) that directly compare CO_2 production or O_2 consumption among treatments. Most insects deplete carbohydrates and utilize lipids when starved (Chown & Nicolson, 2004), so many of these apparent decreases in metabolism may disappear if metabolic rate is compared. In *Drosophila*, starved animals appear to utilize primarily carbohydrates (Marron *et al*., 2003), so comparing metabolic rates instead of CO_2

emission may reveal a still larger decrease in metabolic rate with starvation. Caloric restriction results in a reduction of metabolic rate in *Carausius morosus* stick insects (Roark & Bjorndal, 2009), although the fuels utilized during caloric restriction are uninvestigated, so care must also be taken when comparing metabolic rates in caloric restriction studies. Most of the mass lost is lipid and water, which are nonmetabolizing (Djawdan *et al*., 1997). Thus, using fresh mass as a covariate (as in the present study) may underestimate the metabolic rate of the starved individuals. Accounting for this underestimate would further reduce the difference in metabolic rate between the 1- and 7-day starved crickets in the present study.

In most metabolic studies, feeding status is similar among treatments (or animals are not feeding, in the case of pupae and overwintering stages), so comparisons of CO_2 emission in these cases are likely valid, although Chappell *et al*. (2009) show that RQ may also change with temperature and activity level. However, metabolic rate effects of starvation or specific dynamic action should be conducted with caution with respect to assumed energy utilization. The present study does not measure RQ but estimates it based on patterns of fuel consumption. There are surprisingly few published studies of variation in RQ in insects during stress exposure. Clearly, the measurement of RQ would be valuable for interpreting metabolic changes.

There is no evidence of condition-dependence of metabolic rate in the present study, which suggests that an increase in the efficiency of resource use (by reducing consumption) is not a response to loss of condition, and therefore that condition dependence of other traits in *G. bimaculatus* (Simmons, 1988) is not a direct consequence of an overall strategy to depress metabolic rate at rest. However, measurements on quiescent animals may not reflect their performance when, for example, competing for mates, and it is quite possible that there is no difference in metabolic rate because a basic level of maintenance metabolism is maintained (Makarieva *et al*., 2006). However, no measurements are made of metabolic scope, and it is likely that a reduction in energy reserves from starvation will affect either the maximum metabolic rate or the ability to sustain metabolic output. Changes in metabolic scope would be expected to impact upon costly activities such as singing (Hoback & Wagner, 1997; for a framework that hypothesizes a tight link between resting and maximal metabolic rates among species of insects, see also Reinhold, 1999). Ketola & Kotiaho (2009) do not find any relationship between a measure of metabolic scope and overall condition indices in fed individuals of the cricket *Gryllodes sigillatus*, although they do observe changes in energy allocation, which suggests that the relationship between condition and metabolism may only be manifest over longer periods of the life of the individual.

In conclusion, if changes in fuel utilization are accounted for, metabolic rate in *G. bimaculatus* does not decrease with starvation. This may be general among insects because few studies take fuel utilization into account when comparing metabolic rates among feeding treatments. If condition-dependence of other traits is governed by metabolism, it may be manifest in the value of (or the ability to sustain) high metabolic activity, rather than resting levels.

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