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# Size-dependent energetics of metamorphosis in the yellow dung fly, *Scathophaga stercoraria*

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## ABSTRACT

**Competing hypotheses:** (1) Large body size confers more efficient energy use (relative efficiency hypothesis). (2) Large body size requires more energy to be sustained, a disadvantage when food is limited (absolute energy demand hypothesis).

**Organism:** Yellow dung flies, *Scathophaga stercoraria* (Diptera: Scathophagidae), artificially selected for large and small body size in the laboratory for 24 generations to augment the available phenotypic body sizes.

**Methods:** Larvae were reared under limited and unlimited food (dung) conditions, and the energy content of pupae was measured at the beginning and the end of the pupal stage in different, size-matched individuals.

**Conclusions:** Over the pupal period, lipids and glycogen decreased whereas sugar content increased. Net energy loss per unit body mass was higher at unlimited food. Contrary to expectation, males (the larger sex) lost less energy than females. Large selection line pupae showed the highest absolute and mass-specific energy loss during metamorphosis, indicating a correlated physiological response to body size selection because phenotypic body sizes do not differ between the lines at limited dung. We conclude that energetic costs due to greater absolute energy demand of larger individuals during the pupal phase outweigh the benefits due to greater metabolic efficiency.

*Keywords*: artificial selection, body size, development, energy reserves, insect pupa, life history, metabolic rate, survival.

## INTRODUCTION

The life history and fitness of most organisms are most crucially influenced by body size. There is overwhelming evidence for large body size to be advantageous. In males these advantages are mainly due to sexual selection (male–male competition and/or female choice), whereas in females there is strong fecundity selection favouring larger females (Shine, 1988; Honek, 1993; Andersson, 1994). However, in most organisms size appears to be under stabilizing

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selection (Schluter *et al.*, 1991), which raises the question of what conditions disfavour large individuals and benefit the small (Blanckenhorn, 2000). Generally, large-sized individuals are thought to suffer greater mortality, although empirical evidence for this is limited (Blanckenhorn, 2000). These viability costs can be divided into the costs of becoming large during the juvenile stage, whereby juveniles suffer costs of fast growth (Werner and Anholt, 1993; Gotthard *et al.*, 1994; Blanckenhorn, 1998) or longer development (Roff, 1980; Blanckenhorn, 1998), and the viability and reproductive costs of being a large adult (Madsen and Shine, 1994; Westendorp and Kirkwood, 1998). For example, large individuals might be more susceptible to parasites (Solbreck *et al.*, 1989; Zuk and Kolluru, 1998), parasitoids or predators (Andersson, 1994; Blanckenhorn, 2000). Furthermore, energetic limitations in terms of sustaining large body size have been shown to be disadvantageous under food limitation (Clutton-Brock *et al.*, 1985; Gotthard *et al.*, 1994; Blanckenhorn *et al.*, 1995; Wikelski *et al.*, 1997; Blanckenhorn, 1998). Detailed studies directly examining physiological disadvantages of large size are rare and mostly restricted to the adult life stage.

The allometry between the energy requirements of an organism and body mass was initially described by Kleiber (1932). Studying mammals and birds he found that the mass-specific metabolic rate ($M_b$) decreased with increasing body size by $M_b^{3/4}$ (Kleiber's law). Hemmingsen (1960) expanded this relationship to the whole Animal Kingdom, and more recently West *et al.* (1997) used Kleiber's law to describe the scaling of biological rates with body size in general (see also Heusner, 1982; White and Seymour, 2003; Kozlowski and Konarzewski, 2004). Thus mass-specific metabolic rate generally decreases with increasing body size, and large animals can be said to have a more efficient energy turnover than small animals (reviewed in Glazier, 2005). Although all these studies were concerned with comparisons among species with vastly different body sizes (i.e. the macro-evolutionary level), Kleiber's law in principle also applies at the micro-evolutionary level within species (reviewed by Glazier, 2005; e.g. Nakaya *et al.*, 2005; Reim *et al.*, 2006a), if only for the major tenet of evolution that macro-evolutionary patterns should be grounded in equivalent micro-evolutionary mechanisms. On the other hand, within species there is also some evidence that large individuals need more absolute amounts of energy to sustain their body functions, potentially resulting in higher mortality of large individuals under food shortage (Blanckenhorn *et al.*, 1995; Wikelski *et al.*, 1997; Donohue *et al.*, 2002). That is, large body size confers a lower mass-specific metabolic rate (according to the relative efficiency hypothesis = Kleiber's law), an advantage if sufficient food is available, but large body size requires more energy to be sustained, a disadvantage when food is limited (according to the absolute energy demand hypothesis). These two physiological hypotheses thus yield opposite predictions that are rarely contrasted explicitly in experimental studies within species.

The yellow dung fly *Scathophaga stercoraria* (sometimes *Scatophaga*; Diptera: Scathophagidae) is typical in that large size is strongly favoured by sexual selection in males and by fecundity selection in females (Borgia, 1982; Sigurjónsdóttir and Snorrason, 1995; Jann *et al.*, 2000; Kraushaar and Blanckenhorn, 2002; summarized in Blanckenhorn, 2007). In contrast, it is difficult to find selective disadvantages of large size, a general problem in most animals (Blanckenhorn, 2000, 2007). Blanckenhorn (1998) and Teuschl *et al.* (2007) found that especially when larval resources were limited, large phenotypes and genotypes showed higher pre-adult mortality in the field. Furthermore, towards the end of the season, larger individuals growing up in unlimited dung inducing longer development periods also showed higher pupal mortality due to frost, and generally males, the larger sex in this species, suffered greater winter mortality. As these experiments were conducted in predator-free environments, this suggests physiological costs of large size and/or fast growth (Blanckenhorn, 1998; Teuschl *et al.*, 2007).

A handful of earlier studies directly assessed the energy content of insect pupae (Astaurov, 1957; Bursell, 1961; Stafford, 1973; Hamburger *et al.*, 1996), some of them focusing on a comparison of diapausing and non-diapausing individuals (Tammaru *et al.*, 2001; Ding *et al.*, 2003). However, as a rule these studies did not consider differential effects of body size, and we are not aware of any physiological study in insects specifically addressing size-specific energetics of metamorphosis in a life-history context. Here, we investigate the size-dependent energetic content during the pupal phase in yellow dung flies. We examined yellow dung fly pupae from laboratory body size selection lines, primarily to augment the available phenotypic body sizes so that effects are better detectable (Teuschl *et al.*, 2007). Because a good environment, or good condition, often masks expected life-history costs and trade-offs (van Noordwijk and de Jong, 1986; Alatalo *et al.*, 1990), larvae were reared in limited (i.e. stressful) and unlimited larval food (= dung) conditions, and lipid, glycogen, and sugar contents were measured at the beginning and the end of the pupal period in different, size-matched individuals. We did not measure proteins because our physiological method assesses primarily structural (as opposed to free) proteins (Minari and Zilversmit, 1963), which largely reflect body mass (Blanckenhorn *et al.*, 2007a). We expected the absolutely higher energy consumption of larger flies and the larger males to outweigh their more efficient energy utilization (i.e. Kleiber's law), as evidenced by greater energy loss over the pupal phase, thus demonstrating some physiological costs of large (pupal) size.

## MATERIALS AND METHODS

### Study animal

Yellow dung flies are widespread and abundant in north-temperate regions of the Old and the New World (Stone *et al.*, 1965; Gorodkov, 1984). Females lay their eggs primarily into cattle dung, which the developing larvae feed on and thereby deplete. Dung limitation, typically mediated by high intra- and inter-specific competition, imposes food and time limitations on development (Amano, 1983; Blanckenhorn, 1998). Individuals have to complete larval development to overwinter as pupae (Blanckenhorn, 1998), at which point adult body size is fixed but pupal development (i.e. metamorphosis) still requires time to be completed. Body size in this species is greatly influenced by temperature and the amount of dung individuals could feed on as larvae (Blanckenhorn, 1997, 1998, 1999), both of which vary strongly in space and time, but it also has a heritable component (Simmons and Ward, 1991; Blanckenhorn, 2002).

### Experimental treatments

Flies for this experiment stemmed from the 24th generation of artificial lines selected for large and small adult body size [plus an unselected control (see Teuschl *et al.*, 2007)]. Two generations before this study, the original two replicates of each selection line (small, control, large) were crossed to offset potential inbreeding effects. For this experiment, 20 females (i.e. families) of each of the crossed large, control, and small selection lines were allowed to mate within the line (but otherwise randomly) and lay eggs. The next day the hatched larvae of each clutch were split. Ten larvae were transferred individually into 10 separate containers ($20 \times 20 \times 15$ mm$^3$) with unlimited dung (4–5 g), one larva per container. Twenty other larvae from the same clutch were transferred individually into 20 different 50-ml group containers. This was done for all 20 families, so that each group

container hosted larvae from 20 different families under limited dung conditions [5–6 g per container (cf. Amano, 1983)]. Note that because larvae are so small and dung tends to dry quickly, limited dung conditions could not be effectively administered to single larvae, hence the group rearing treatment. This (potential) stock of 200 (20 families × 10) larvae under unlimited dung plus 400 (20 families × 20) larvae under limited dung conditions was sufficiently generous to meet our targeted sample size in the face of expected larval mortality.

After 10 days of larval development at 20°C, the fresh weight of a total of 72 pupae of each line (i.e. 3–4 per family) and dung treatment was measured at the *beginning* of the pupal period. From each line and dung treatment, 32 of these 72 pupae were then fixated in 100% ethanol and heated for approximately 10 min at 90°C for subsequent energy analysis (described below). Under unlimited dung conditions, 72 pupae of the control and small selection lines were similarly weighed and 32 pupae fixated for energy analysis at the *end* of the pupal period, 19 days after the eggs were laid; 32 pupae of the large line were treated likewise on day 21, as their development time was known to be longer (Teuschl *et al.*, 2007). Under limited dung conditions, development time is shortened (Blanckenhorn, 1998, 1999), so pupae at the end were analysed one day earlier at day 18 (control and small line) and day 20 (large line). These end-of-pupal-period estimates were empirically derived from our knowledge of line-specific development times (Teuschl *et al.*, 2007). We could not easily apply the same adjustment with regard to the sexes, whose development times also differ systematically but overlap within lines, although we controlled for this statistically as described below. We are well aware of the problems introduced by these variable pupal periods, but note that the use of a fixed pupal period for all individuals instead would have implied rather different physiological end points for the various treatment combinations: a no-win situation. Given inevitable systematic variation in development times, our procedure best approximates estimation of the whole pupal period, the biologically relevant entity in the life-history context we were interested in.

Pupae not used for energy analysis were further assigned to two different treatments. Some of the pupae weighed at the beginning were weighed a second time at the end of the pupal period to estimate potential pupal weight (i.e. water or energy) loss during metamorphosis, which could then be controlled for later. The remaining pupae were dried for 3 days at 60°C to estimate the water content and dry mass of yellow dung fly pupae, some at the beginning and others at the end of the pupal period, again for the purposes of *post-hoc* statistical control (described below).

## Energy analysis

The pupae's energy content was analysed by the photometric method of Van Handel and Day (1988). Flies were homogenized individually in 250 $\mu$l of 2% $NaSO_4$, which absorbs and precipitates the glycogen while the soluble oligosaccharides were extracted by adding a 1 : 1 chloroform/methanol mixture. Interfering chitin that co-precipitates with glycogen was dissolved by 30% KOH (for 10 min at 90°C) and discarded after centrifugation. Haemolymph trehalose and crop content of various sugars were not separated in our analyses because we were interested in the total caloric amount of oligosaccharides as energy substrates for utilization. Glycogen and soluble carbohydrates (henceforth sugars) were quantified photometrically by anthrone reaction with 0.1% glucose in 25% EtOH as the standard (Van Handel, 1985a), and lipids following the vanillin reaction with 0.1% soybean oil

in chloroform as the standard (Van Handel, 1985b). To facilitate direct comparisons, the values of all components were converted to calories, where 1 calorie = 4.187 Joules, corresponding to 250 $\mu$g of carbohydrates or protein and 110 $\mu$g of lipids.

## Statistical methods

For the subset of pupae weighed twice, weight differences (comprising water and energy loss) between the beginning and the end of the pupal period were analysed using repeated-measures analysis of variance (ANOVA). Subsequently, the fresh weights of all the pupae of the main sample weighed and analysed only at the end were corrected by adding this estimated overall mean weight reduction between the beginning and end (assuming it was largely due to water loss).

We first tested for fresh and dry weight differences between the beginning and the end of the pupal period using univariate ANOVA with selection line, pupal status (beginning and end), and dung treatment as fixed factors. Fresh weight of the pupae measured at the end was corrected for water loss in this analysis, as described above, but dry weight was not so corrected. In all analyses, fresh and dry weights and all energy components, which all represent volumes, were cube-root-transformed so as to analyse linear equivalents, which typically better follow a normal distribution. Differences in sugar, glycogen, and lipid contents between beginning and end of the pupal period were investigated using analogous univariate analyses of covariance (ANCOVA) with selection line and pupal stage (beginning or end) as fixed factors and fresh weight as a covariate (raw for the beginning, corrected as explained above for the end value). This analysis was performed separately for unlimited and limited dung, as sex could only be included as an additional fixed factor for unlimited dung. This is because under limited dung conditions the sex of pupae could not be determined from fresh weight, as flies did not differ in weight or size, whereas under unlimited dung conditions males were heavier and larger than females and also required 1–2 days more for development, yielding non-overlapping size distributions for the sexes (Teuschl *et al.*, 2007 and unpublished data).

As all pupae of a given selection line were measured after the same period of time at the end of the pupal period, the energy content of male pupae reared under unlimited dung conditions had to be statistically corrected for their 0.5–2 day longer pupal period, because under unlimited conditions male pupae would have consumed energy for up to two more days (cf. above) (Teuschl *et al.*, 2007). This was done by adjusting the actual end value by the corresponding number of daily energy equivalents, separately for lipids, glycogen, and sugars. The daily energy equivalent was estimated, for each component, as the total mean difference between the values measured at the beginning and the end divided by the mean pupal period, thus assuming equal energy consumption on all days. This correction could also be applied *post-hoc* to adjust all selection line × dung treatment × sex combinations according to their actual mean development times. Note that different individuals were measured at the beginning and the end of metamorphosis, as the energy content cannot be measured twice for the same individual; consequently, we did not use repeated-measures ANOVA. Because individuals were randomly assigned, no systematic differences between the beginning and end values were expected other than those we tested for.

A final analysis served to investigate differences between the lines (and the sexes) in the total energy loss or gain (in calories) between beginning and end of pupation by integrating all three energy components. For glycogen, sugars, and lipids, separate linear regressions

were performed for the dependence on a pupa's energy content (measured at the beginning of metamorphosis) on fresh weight. From this relationship we could estimate the putative starting point of those pupae of a given corrected weight for which the energy content was measured at the end of the pupal stage. For each individual, the change in energy content was then separately calculated for the three energy components by taking the difference between the estimated (i.e. presumed) energy content at the beginning and the measured, weight-corrected energy content at the end of the pupal stage. The total energy change (in calories) over the entire pupal period was then calculated by summing these differences for all three energy components. This value was again cube-root-transformed as for volumes, and total energy change during the pupal stage was tested for differences among selection lines using univariate ANCOVA with (corrected) cube-root fresh weight as the covariate (as above). Again, this analysis was performed separately for limited and unlimited dung so that sex could also be included in the latter.

## RESULTS

Fresh weight and dry weight of pupae correlated strongly (all treatments and sexes combined: $r = 0.99$, $P < 0.001$, $n = 359$, $y_{(\text{fresh weight in mg})} = 1.44x_{(\text{dry weight in mg})} + 0.278$). The percentage of water of a pupa's fresh weight was $74.1 \pm 0.4\%$ ($\pm$S.E.) for pupae from the unlimited dung condition and $78.1 \pm 0.3\%$ for pupae from the limited dung condition.

For those pupae weighed twice (unlimited food: $n = 42$; limited food: $n = 30$), the weight difference between the beginning (mean $22.04 \pm 1.40$ mg) and the end of the pupal period (mean $21.30 \pm 1.40$ mg) was overall highly significant (repeated-measures analysis: $F_{1,71} = 253.30$, $P < 0.001$). The fresh weight of all pupae measured at the end of the pupal period was consequently corrected upward by the mean difference of 0.74 mg (3.4%; presumed to be water loss) when used as a covariate in all further analyses.

The changes in contents of lipid, sugars, and glycogen between the beginning and the end of the pupal period are shown in Table 1, separately for the selection lines, sexes, and dung treatments. Note that the data in Table 1 are raw values not corrected for size, while all data shown in Figs. 1–4 are corrected for fresh pupal weight after ANCOVA, as all three energy components increased markedly with fresh weight ($y_{(\text{lipids})} = 0.80x_{(\text{fresh weight})} - 0.31$, $r = 0.94$; $y_{(\text{glycogen})} = 0.34x_{(\text{fresh weight})} - 0.12$, $r = 0.77$; $y_{(\text{sugars})} = 0.22x_{(\text{fresh weight})} + 0.05$, $r = 0.80$) (Table 2). In both dung conditions, lipid and glycogen contents generally decreased during metamorphosis, likely reflecting consumption (pupal stage effect in Table 2; see Table 1). Interestingly, sugar content showed an overall increase (significant pupal stage effect in Table 2), as presumably part of the glycogen metabolized was mobilized in the form of haemolymph sugars (Table 1; Fig. 1). Additionally, large line flies showed a greater loss of lipids and glycogen but also greater sugar gains (Fig. 1; line $\times$ pupal stage interaction in Table 2), and at least the latter was more pronounced at unlimited than limited dung (Fig. 1; Table 2).

Under unlimited dung, males overall lost fewer lipids than females during the pupal stage, even though all three male energy components were conservatively corrected *post-hoc* for their longer development time (Fig. 2; sex $\times$ pupal stage interaction in Table 2). Even though lipid and glycogen contents at the beginning of the pupal period appeared equal in both sexes, overall there was a sex effect because females had lost more by the end (Fig. 2; Table 2). Moreover, males gained more sugars than females (Fig. 2; sex $\times$ pupal stage interaction in Table 2), while glycogen loss did not differ between the sexes. These changes

**Table 1.** Energy content (in calories) and fresh weight of pupae at the beginning and the end of the pupal period, separately for food (dung) treatment, selection line, and sex (mean ± S.E.)

| Condition | | | Pupal stage | |
|---|---|---|---|---|
| | | | Start | End |
| **Unlimited dung** | | | | |
| Large | males | lipids (cal) | 16.84 ± 0.33 | 9.71 ± 0.62 |
| | | glycogen (cal) | 1.70 ± 0.06 | 0.45 ± 0.07 |
| | | sugars (cal) | 0.34 ± 0.03 | 1.12 ± 0.15 |
| | | total energy (cal) | 18.87 ± 0.32 | 11.25 ± 0.77 |
| | | fresh weight (mg) | 46.87 ± 0.95 | 39.38 ± 0.62 |
| | females | lipids (cal) | 11.80 ± 0.29 | 6.12 ± 0.27 |
| | | glycogen (cal) | 1.04 ± 0.04 | 0.20 ± 0.03 |
| | | sugars (cal) | 0.28 ± 0.01 | 0.30 ± 0.04 |
| | | total energy (cal) | 13.12 ± 0.32 | 6.61 ± 0.32 |
| | | fresh weight (mg) | 26.99 ± 0.29 | 24.33 ± 0.51 |
| Control | males | lipids (cal) | 13.60 ± 0.45 | 9.77 ± 0.28 |
| | | glycogen (cal) | 1.51 ± 0.05 | 0.59 ± 0.04 |
| | | sugars (cal) | 0.33 ± 0.01 | 0.59 ± 0.03 |
| | | total energy (cal) | 15.44 ± 0.48 | 10.93 ± 0.31 |
| | | fresh weight (mg) | 36.05 ± 0.63 | 33.55 ± 0.51 |
| | females | lipids (cal) | 8.79 ± 0.25 | 3.93 ± 0.39 |
| | | glycogen (cal) | 0.81 ± 0.03 | 0.18 ± 0.05 |
| | | sugars (cal) | 0.31 ± 0.01 | 0.21 ± 0.04 |
| | | total energy (cal) | 9.90 ± 0.28 | 4.33 ± 0.41 |
| | | fresh weight (mg) | 21.25 ± 0.39 | 19.39 ± 0.80 |
| Small | males | lipids (cal) | 12.31 ± 0.36 | 8.63 ± 0.35 |
| | | glycogen (cal) | 1.25 ± 0.05 | 0.39 ± 0.05 |
| | | sugars (cal) | 0.30 ± 0.01 | 0.49 ± 0.04 |
| | | total energy (cal) | 13.86 ± 0.40 | 9.51 ± 0.40 |
| | | fresh weight (mg) | 30.48 ± 0.72 | 26.95 ± 0.51 |
| | females | lipids (cal) | 7.41 ± 0.20 | 5.28 ± 0.35 |
| | | glycogen (cal) | 0.68 ± 0.02 | 0.23 ± 0.03 |
| | | sugars (cal) | 0.26 ± 0.01 | 0.25 ± 0.02 |
| | | total energy (cal) | 8.36 ± 0.21 | 5.76 ± 0.39 |
| | | fresh weight (mg) | 16.54 ± 0.51 | 15.66 ± 0.64 |
| **Limited dung** | | | | |
| Large | | lipids (cal) | 2.32 ± 0.10 | 1.20 ± 0.55 |
| | | glycogen (cal) | 0.29 ± 0.01 | 0.06 ± 0.06 |
| | | sugars (cal) | 0.10 ± 0.00 | 0.14 ± 0.11 |
| | | total energy (cal) | 2.71 ± 0.11 | 1.40 ± 0.69 |
| | | fresh weight (mg) | 8.23 ± 0.22 | 8.35 ± 1.24 |
| Control | | lipids (cal) | 2.25 ± 0.09 | 1.67 ± 0.59 |
| | | glycogen (cal) | 0.32 ± 0.01 | 0.10 ± 0.06 |
| | | sugars (cal) | 0.10 ± 0.01 | 0.12 ± 0.04 |
| | | total energy (cal) | 2.68 ± 0.10 | 1.90 ± 0.67 |
| | | fresh weight (mg) | 7.34 ± 0.19 | 7.97 ± 1.39 |
| Small | | lipids (cal) | 3.16 ± 0.12 | 1.62 ± 0.43 |
| | | glycogen (cal) | 0.32 ± 0.02 | 0.06 ± 0.04 |
| | | sugars (cal) | 0.10 ± 0.00 | 0.12 ± 0.03 |
| | | total energy (cal) | 3.59 ± 0.14 | 1.80 ± 0.49 |
| | | fresh weight (mg) | 8.42 ± 0.27 | 7.46 ± 0.89 |

*Note*: Fresh weight of pupae at the end was corrected for water and energy loss by adding 0.74 mg, and energy components were corrected for pupal period duration (see text; $n = 28$–$32$ per treatment combination).

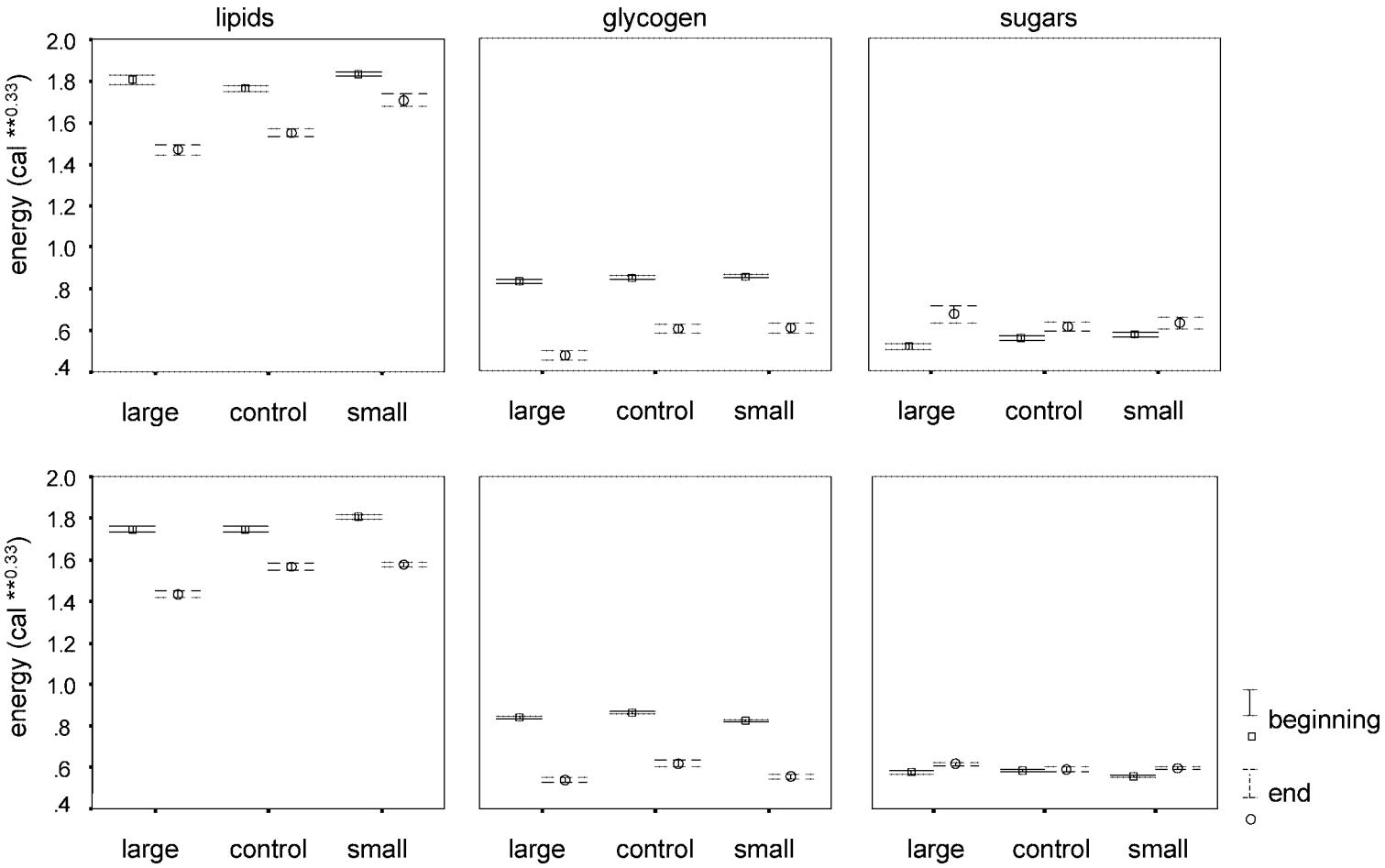


**Fig. 1.** Mean lipid, glycogen, and sugar content (±S.E.) at the beginning and the end of the pupal period for three selection lines under different dung conditions (top, unlimited dung; bottom, limited dung; data corrected for fresh body weight; both sexes combined).

**Table 2.** Results of univariate ANCOVAs of cube-root-transformed lipid, glycogen, and sugar content with selection line and pupal stage (beginning or end) as fixed factors and fresh pupal weight as covariate, for unlimited dung (top: with sex as an additional fixed factor) and limited dung (bottom)

| | | Lipids | | | Glycogen | | | Sugars | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | d.f. | MS | *F* | *P* | MS | *F* | *P* | MS | *F* | *P* |
| **Unlimited dung** | | | | | | | | | | |
| Intercept | 1 | 0.161 | 17.561 | <0.001 | 0.030 | 4.890 | <0.001 | 0.000 | 0.038 | <0.001 |
| Line | 2 | 0.066 | 7.144 | 0.001 | 0.005 | 0.867 | 0.422 | 0.019 | 2.770 | 0.066 |
| Pupal stage | 1 | 2.713 | 295.655 | <0.001 | 3.635 | 594.146 | <0.001 | 0.359 | 52.207 | <0.001 |
| Sex | 1 | 0.102 | 11.066 | 0.001 | 0.072 | 11.719 | 0.001 | 0.002 | 0.285 | 0.594 |
| Fresh weight | 1 | 0.363 | 39.599 | <0.001 | 0.058 | 9.415 | 0.003 | 0.128 | 18.613 | <0.001 |
| Line × pupal stage | 2 | 0.149 | 16.189 | <0.001 | 0.078 | 12.690 | <0.001 | 0.069 | 10.024 | <0.001 |
| Line × sex | 2 | 0.082 | 8.898 | <0.001 | 0.021 | 3.439 | 0.034 | 0.027 | 3.895 | 0.022 |
| Pupal stage × sex | 1 | 0.097 | 10.589 | 0.001 | 0.002 | 0.398 | 0.529 | 0.681 | 99.038 | <0.001 |
| Line × pupal stage × sex | 2 | 0.060 | 6.568 | 0.002 | 0.008 | 1.358 | 0.260 | 0.034 | 4.942 | 0.008 |
| Error | 161 | 0.009 | | | 0.006 | | | 0.007 | | |
| **Limited dung** | | | | | | | | | | |
| Intercept | 1 | 0.002 | 0.418 | 0.519 | 0.003 | 0.851 | 0.358 | 0.003 | 1.820 | 0.179 |
| Line | 2 | 0.180 | 34.749 | <0.001 | 0.050 | 14.074 | <0.001 | 0.006 | 3.361 | 0.037 |
| Pupal stage | 1 | 2.658 | 513.930 | <0.001 | 3.451 | 977.081 | <0.001 | 0.034 | 18.740 | <0.001 |
| Fresh weight | 1 | 1.411 | 272.784 | <0.001 | 0.301 | 85.295 | <0.001 | 0.145 | 78.190 | <0.001 |
| Line × pupal stage | 2 | 0.071 | 13.764 | <0.001 | 0.012 | 3.317 | 0.038 | 0.006 | 3.010 | 0.052 |
| Error | 181 | 0.005 | | | 0.004 | | | 0.002 | | |

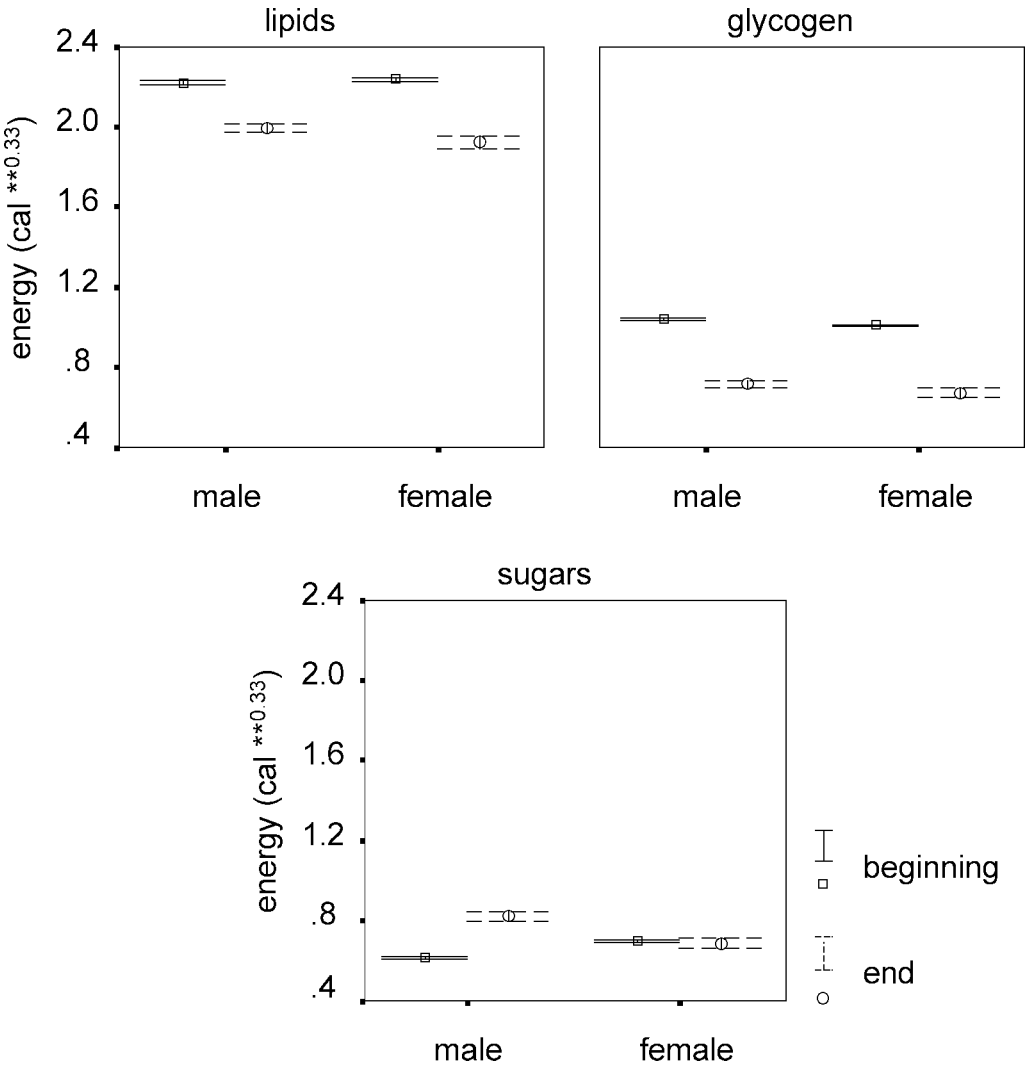


**Fig. 2.** Mean lipid, glycogen, and sugar content (±S.E.) under unlimited dung conditions at the beginning and end of the pupal period for males and females (data are corrected for body weight; selection lines and dung treatments combined).

further differed between the three selection lines (being greatest for large line flies), resulting in three-way (line × sex × pupal stage) interactions for lipids and sugars but not glycogen (Fig. 3; Table 2). Overall, more lipid consumed combined with less sugar synthesized by females suggests that metamorphosis might be energetically more costly for females than males. Moreover, while female lipid and glycogen contents varied inconsistently between the lines, large line males showed consistently lower values (line effects and sex × line interactions in Table 2; Fig. 3). In contrast, large line males had the highest sugar contents at the end of the pupal period, whereas in females this was the case for small line individuals (line effect and sex × line interaction in Table 2; Fig. 3).

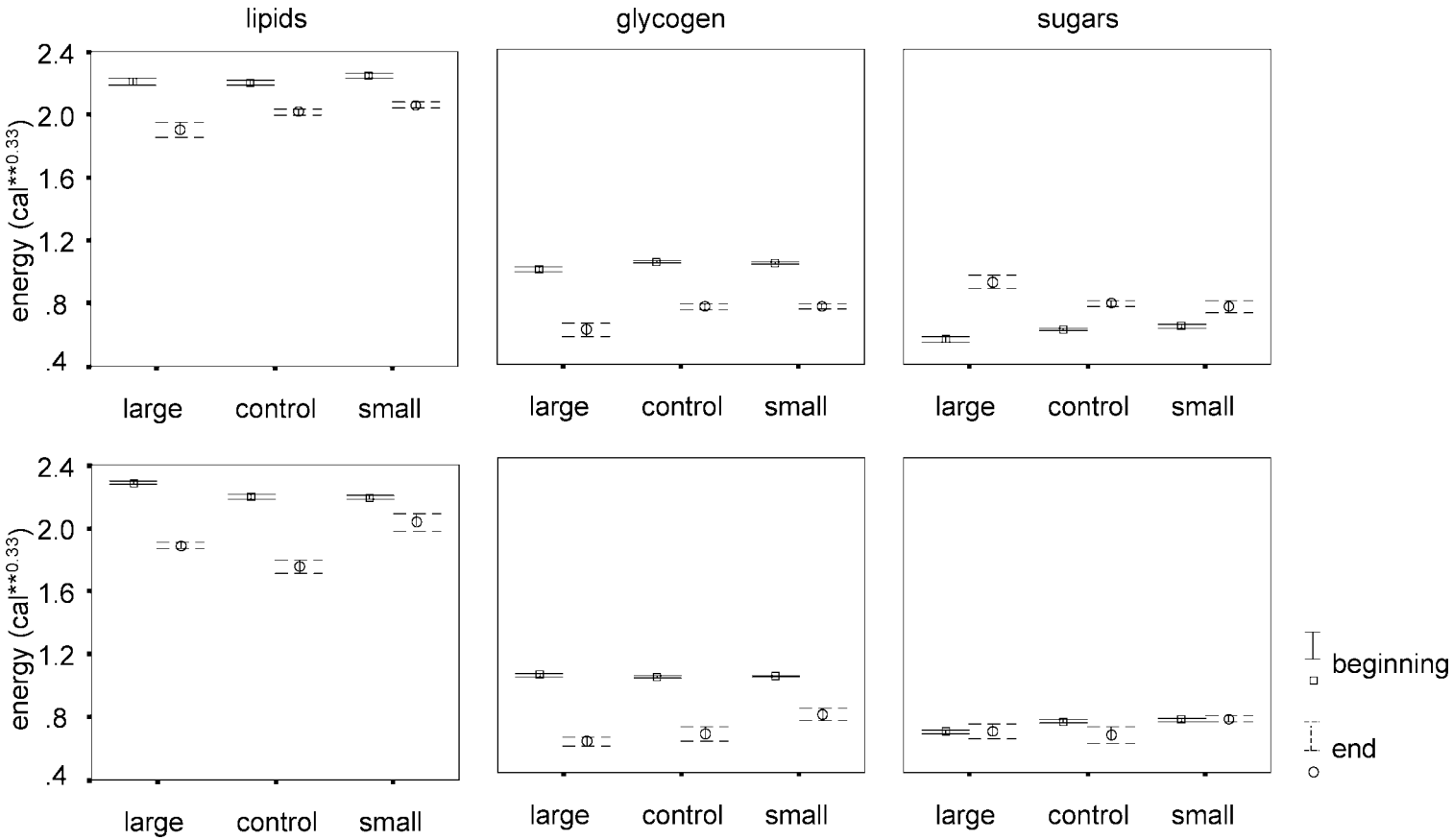


**Fig. 3.** Mean lipid, glycogen, and sugar content (±S.E.) under unlimited dung conditions at the beginning and end of the pupal period for three selection lines and the sexes (top, males; bottom, females; data are corrected for body weight; both dung treatments combined).

Taking the differences between the beginning and end values for all three energy components and adding them up, we were able to analyse the overall size-corrected [i.e. structural-protein corrected (Blanckenhorn *et al.*, 2007a)] net energy changes of individuals (Fig. 4). These values are very close to the values for lipids in Figs. 1–3 because the caloric equivalent for lipids is about two times higher than that for carbohydrates, and because glycogen loss and sugar gain largely cancel out. Under both unlimited and limited dung, flies of the large selection line lost most energy (unlimited dung: $\text{Contrast}_{\text{(large vs. control)}}$: $t_{56} = 1.786$, $P = 0.078$; $\text{Contrast}_{\text{(control vs. small)}}$: $t_{56} = 3.095$, $P = 0.003$; limited dung: $\text{Contrast}_{\text{(large vs. control)}}$: $t_{59} = 3.805$, $P < 0.001$; $\text{Contrast}_{\text{(control vs. small)}}$: $t_{59} = 0.125$, $P = 0.900$) (Fig. 4; Table 3). Large line individuals lost more even under limited dung despite negligible size differences between the selection lines (Table 1; Fig. 4). This suggests that the higher energy loss (i.e. consumption) of large line flies is a correlated response to body size selection, as Figs. 1–4 all present size-corrected (i.e. mass-specific) values.

Finally, under limited but not unlimited dung, energy loss (consumption) increased significantly with fresh weight (i.e. body size) when statistically controlling for selection

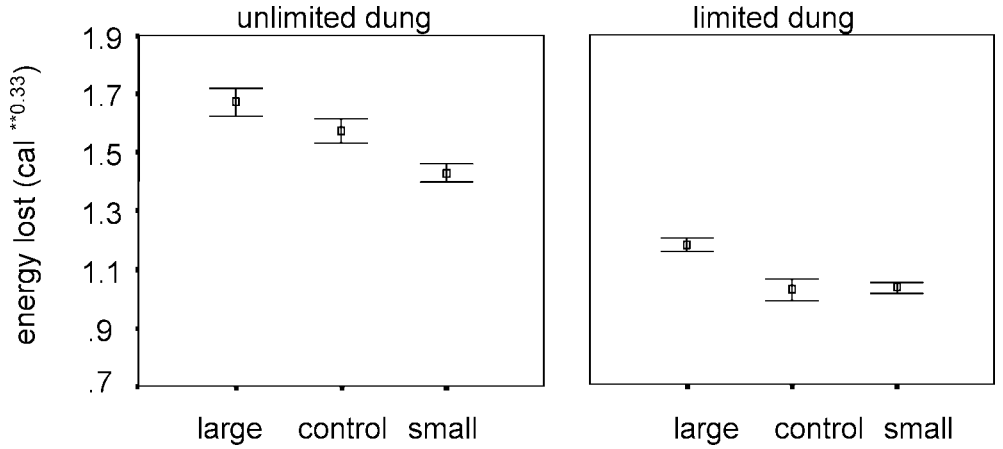


**Fig. 4.** Mean overall energy (lipids, glycogen, sugars) lost (±S.E.) during the pupal phase in the unlimited and limited dung conditions (data are corrected for body weight; sex ignored).

**Table 3.** Results of univariate ANCOVAs of total net pupal energy consumption with line as fixed factor and fresh weight as covariate for unlimited dung (with sex as an additional fixed factor) and limited dung

| | Unlimited dung | | | | Limited dung | | | |
|---|---|---|---|---|---|---|---|---|
| | d.f. | MS | *F* | *P* | d.f. | MS | *F* | *P* |
| Intercept | 1 | 0.251 | 7.013 | 0.010 | 1 | 0.009 | 0.351 | 0.555 |
| Line | 2 | 0.224 | 6.248 | 0.003 | 2 | 0.223 | 9.003 | <0.001 |
| Sex | 1 | 0.001 | 0.023 | 0.879 | | | | |
| Fresh weight | 1 | 0.004 | 0.122 | 0.728 | 1 | 0.343 | 13.833 | <0.001 |
| Line × sex | 2 | 0.065 | 1.817 | 0.170 | | | | |
| Error | 79 | 0.036 | | | 87 | 0.025 | | |

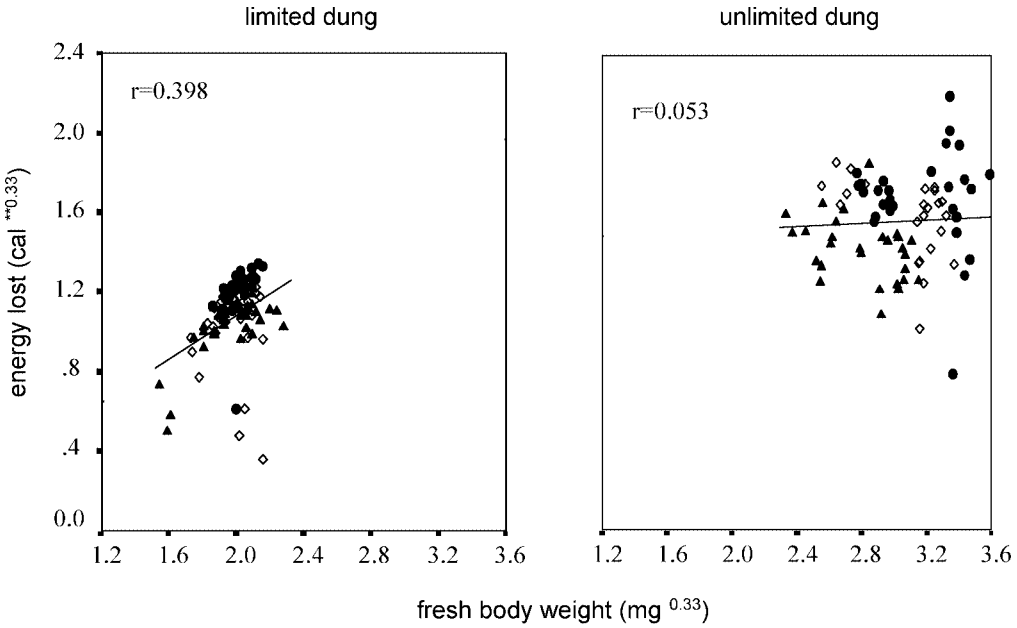


**Fig. 5.** Relationship between individual net energy loss during the pupal phase and fresh body weight under limited (left; Y-intercept not different from zero) and unlimited (right) dung conditions for the small (▲), control (◇), and large (●) selection lines (sex ignored).

line (Fig. 5: $y = 0.552x - 0.019$, $r = 0.40$; Table 3). This occurred because under unlimited conditions, dung body size variance within lines was small relative to that between lines. This also suggests that the disadvantages of large body size are most prominent under unfavourable conditions of food stress. Nevertheless, when combining the phenotypic data of all selection lines, food treatments, and the sexes, energy consumption overall increased with body size (cf. Fig. 5).

## DISCUSSSION

Our study of changes in lipids, glycogen, and sugars during the pupal stage in yellow dung flies supports the absolute energy demand hypothesis rather than the relative metabolic efficiency hypothesis, as larger flies and flies of the large selection line showed higher net mass-specific energy loss during the pupal life stage, thus indicating metabolic costs of large size (Fig. 4). As in the pupal stage no energy is taken up, all energy lost must either have been metabolized or reallocated to other compounds (e.g. sugars, as shown here) or body structures during metamorphosis. These two possibilities could not be distinguished using our methods. However, any energy reserves used to build up tissue (e.g. muscles) should have affected body mass and were hence accounted for by our analysis. Although we did not measure proteins here, structural proteins strongly reflect body mass in this and other species (Minari and Zilversmit, 1963; allometries given in Blanckenhorn *et al.*, 2007a), so we only missed free proteins in our analyses, of which there are relatively few. We therefore conclude that although there is evidence for greater metabolic efficiency of larger individuals (Kleiber's, 1932, law) in terms of starvation resistance and adult reproduction in this species (Reim *et al.*, 2006a,

2006b), for pupae this was apparently outweighed by the greater energy demand of larger individuals.

Elevated energy consumption during metamorphosis is expected to be disadvantageous for large yellow dung flies at least in highly competitive situations, and could mediate the higher mortality of pupae under adverse conditions previously shown (Blanckenhorn, 1998), although we cannot establish a direct link. Teuschl *et al.* (2007) found that pupal mortality under limited dung conditions and preferred temperatures (20°C) was overall low (ca. 1.5%) but was about three times higher in the large than the small selection line. This would be consistent with our physiological results here. In heat-stressed (25°C) flies, pupal mortality increased dramatically, but was much higher in the small (ca. 45%) than the large (ca. 27%) selection line. This is inconsistent with our results, although it is likely that mechanisms other than mere starvation, for example desiccation or insufficient heat dissipation, can account for this result (discussed in Blanckenhorn, 2000; Teuschl *et al.*, 2007). In contrast, greater mortality due to frost at the end of the season in this species is likely a consequence of longer larval development times associated with larger size, rather than metabolic costs of producing a larger body *per se* (Blanckenhorn, 1998; Teuschl *et al.*, 2007). In any case, as the emerging adult flies have to feed on sugar and prey to survive [anautogeny (Foster, 1967)], any energetic advantages accruing during the pupal stage will only affect initial adult survival, and Reim *et al.* (2006a) showed clearly that large size increases survival of starved freshly emerged adults due to their greater absolute amounts of energy reserves, thus in this case favouring the energy efficiency hypothesis. Net metabolic costs of large size during the pupal stage also do not necessarily imply similar costs during the preceding larval phase. On the one hand, pupal size will depend on the preceding larval size, so larval metabolic costs are likely proportional. On the other hand, it is known that the allometric relationship of whole-organism metabolism with body size varies with the developmental stage in several organisms (Wieser, 1984; de Souza and Kuribara, 2006). Furthermore, the larval period is the feeding phase, and larger larvae may be more efficient at foraging, energy acquisition or energy conversion (cf. Kause *et al.*, 1999), possibly rendering efficiency advantages due to Kleiber's (1932) law or other effects more important at this stage. Unfortunately, larval metabolism cannot be easily assessed in yellow dung flies.

Although we cannot differentiate between anabolic and catabolic processes using our measurement technique, our data demonstrate size-dependent net consumption of lipids and glycogen during metamorphosis. This is in line with other insect studies (Fink, 1925; Bursell, 1961; Wigglesworth, 1965; Stafford, 1973; Ding *et al.*, 2003) showing that lipid and glycogen contents decrease during the larval and/or pupal phase. For honeybee workers, it has been shown that 90% of the glycogen and 75% of the lipid stored is used for metamorphosis (Astaurov, 1957). Unfortunately, these early studies did not investigate sugars separately, but Stafford (1973) showed for tsetse flies that glycogen content decreased while free sugars increased towards the end of the pupal period. The transformation of glycogen into glucose for flight or when starved is well known in the Diptera (Mason *et al.*, 1989; Graves *et al.*, 1992; Yuval *et al.*, 1994; Warburg and Yuval, 1996; Timmermann and Briegel, 1999; Auerswald and Gade, 2000; Briegel *et al.*, 2001; Marron *et al.*, 2003). Moreover, having short-term energy metabolites such as glucose disposable at the end of the pupal period might be crucial for successful adult emergence. The novel and central result here is that lipid and glycogen consumption and sugar mobilization were shown to be body size dependent (cf. Blanckenhorn, 2000). To complete the picture, measurements of non-structural (i.e. free) proteins using different physiological methods would be useful [structural proteins of adults were assessed by Blanckenhorn *et al.* (2007a)], although we have

no reason to believe that results for free proteins would invalidate the results for the other energy compounds reported here.

While our results indicate that the efficiency advantages of large pupae due to Kleiber's law are small compared with the disadvantages incurred by a greater absolute energy demand during metamorphosis, this does not mean that energy efficiency is not important at this stage. Flies of all lines consumed more energy per unit body mass when resources were plentiful than when they were limited (Fig. 4), and energy consumption only increased with body size as long as resources were limited (Fig. 5). This supports a phenomenon previously highlighted by several authors that presumed costs or trade-offs likely become visible only under stressful environments because individuals can invest maximally in all traits when environmental conditions are good (van Noordwijk and de Jong, 1986; Alatalo *et al.*, 1990).

We did not observe a large reduction in body mass (3.4%) between beginning and end of the pupal period. This can reflect both water and energy loss, which we cannot separate, although we assume it is mostly water loss. Odell (1998) points out that most larval tissues become reorganized during metamorphosis, so the comparably small energy consumption need not necessarily show up in a large weight loss.

Contrary to expectation, under unlimited dung conditions the larger males did not lose more but rather fewer lipids and at the same time mobilized more sugars than females during metamorphosis (Fig. 2). However, mass-specific total energy consumption (i.e. the sum of all three individual energy components) did no longer differ between the sexes, indicating that differences between the sexes in consumption of lipids and glycogen and synthesis of sugars cancel out. Systematic physiological differences between the sexes can be expected and have been widely reported in insects (e.g. Warburg and Yuval, 1996; O'Brien, 1999; Rogowitz and Chappell, 2000; Kurtz and Sauer, 2001; Krasnov *et al.*, 2002; Schwarzenbach *et al.*, 2004) and other animals (Roeder *et al.*, 1990; Yang and Gordon, 1996; Burley and Foster, 2004; Sheel *et al.*, 2004). Our results were unexpected because male yellow dung flies usually require more time for development and typically show greater pre-adult mortality in the field and the laboratory (Blanckenhorn, 1997, 1998). Greater male lipid consumption during metamorphosis was further expected because in holometabolous insects, male gonads begin to develop before those of females during the larval and pupal phases, and gonad production is thought to be costly in terms of development time (Kerkis, 1931; Pitnick *et al.*, 1995; Reed and Beckage, 1997; Dixon, 2000; Blanckenhorn *et al.*, 2007b). Fischer *et al.* (2004) recently showed that in a protandrous butterfly the smaller but faster growing males indeed have higher metabolic rates than females, the opposite of what we found, although their respirometric methods were superior in this context because they assessed catabolism directly rather than a combination of anabolism and catabolism, as we did.

One methodological problem we had was that the energy content of males may not necessarily have been measured at the very end of their pupal period because they have longer development times than females, although we did statistically adjust for this difference *post-hoc* (see Methods). In so doing, we assumed constant energy consumption over time, which likely does not hold. Nevertheless, even if taking into account that energy consumption might follow a U-shaped convex relationship with time, as described for pupal metabolism in several insects (Taylor, 1927; Wigglesworth, 1949; Rajagopal and Bursell, 1965; Penttinen and Holopainen, 1995), and conservatively adjusting for a 3 day rather than the normal 1–2 day development time difference between the sexes (cf. Blanckenhorn, 1997, 1998, 1999), males still did not consume more lipids than females but mobilized even more sugars. If sugar reserves at the end of the pupal period indeed facilitate successful adult emergence, this indicates greater

male investment, perhaps to compensate for their higher probability of dying during adult eclosion (Blanckenhorn, 1998). Alternatively, higher sugar reserves at the end of the pupal period may increase survivorship during initial adulthood. However, longevity of freshly emerged adults was greater for females when adjusting for the body size differences between the sexes (Reim *et al.*, 2006a). In summary, the metabolic advantages during metamorphosis of males relative to females observed here are inconsistent with the generally higher male mortality found in the field and laboratory (Blanckenhorn, 1998, 2007). With regard to our methods, we are confident that our variable end points and the statistical adjustments did not introduce systematic biases so as to invalidate our results and conclusions: when statistically adjusting the data of all treatment combinations (whenever necessary) according to their mean development times (taken from Teuschl *et al.*, 2007) using the estimated daily energy equivalent technique, all results remained qualitatively the same.

In conclusion, the evidence presented here indicates higher absolute *and* relative (i.e. mass-specific) energy costs during metamorphosis for larger individuals of both sexes and for flies artificially selected to be large. We are aware that our selection line results are unreplicated, although the crossed lines generally did not differ qualitatively from the two pure replicate lines for other life-history variables (Teuschl *et al.*, 2007). Nevertheless, the phenotypic results remain valid. These higher metabolic costs might well be the proximate cause of the greater mortality in nature of larger flies and the larger males, although this cannot be proven directly, and pupal (as opposed to larval or adult) mortality is generally low unless the animals are heat stressed (Blanckenhorn, 1998; Teuschl *et al.*, 2007). Whether our results can be replicated in other holometabolous insects or other taxa with complex life cycles [e.g. anurans (cf. de Souza and Kuribara, 2006)] remains to be determined.

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