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Inbreeding effects on standard metabolic rate investigated at cold, benign and hot temperatures in *Drosophila melanogaster*

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ABSTRACT

Inbreeding increases homozygosity, which is known to affect the mean and variance of fitness components such as growth, fecundity and mortality rate. Across inbred lines inbreeding depression is typically observed and the variance between lines is increased in inbred compared to outbred lines. It has been suggested that damage incurred from increased homozygosity entails energetic cost associated with cellular repair. However, little is known about the effects of inbreeding on standard metabolic rate. Using stop-flow respirometry we performed repeated measurements of metabolic rate in replicated lines of inbred and outbred *Drosophila melanogaster* at stressful low, benign and stressful high temperatures. The lowest measurements of metabolic rate in our study are always associated with the low activity period of the diurnal cycle and these measurements therefore serve as good estimates of standard metabolic rate. Due to the potentially added costs of genetic stress in inbred lines we hypothesized that inbred individuals have increased metabolic rate compared to outbred controls and that this is more pronounced at stressful temperatures due to synergistic inbreeding by environment interactions. Contrary to our hypothesis we found no significant difference in metabolic rate between inbred and outbred lines and no interaction between inbreeding and temperature. Inbreeding however effected the variance; the variance in metabolic rate was higher between the inbred lines compared to the outbred control lines with some inbred lines having very high standard metabolic rate. Thus genetic drift and not inbreeding *per se* seem to explain variation in metabolic rate in populations of different size.

1. Introduction

Inbreeding, the reproduction between closely related individuals sharing a recent common ancestor, is an important biological phenomenon in both evolutionary- and conservation biology as well as in animal- and plant breeding (Allendorf et al., 2013; Charlesworth and Charlesworth, 1987; Frankham et al., 2010; Kristensen and Sørensen, 2005). A consequence of severe inbreeding is inbreeding depression which is manifested as a decrease in the mean value of fitness traits, such as growth, fecundity and mortality rate (Crow and Kimura, 1970; Falconer and Mackay, 1996; Kristensen and Sørensen, 2005; Lynch and Walsh, 1998; Reed et al., 2012). Apart from affecting the mean value, inbreeding and genetic drift also result in changes in the genetic variance structure within and between populations, observed as a decrease in heterozygosity and loss and fixation of alleles within populations and an increased genetic differentiation between populations (Falconer and Mackay, 1996). Thus inbreeding and genetic drift are expected to decrease the genetic variance within populations but increase the genetic variance between populations. Inbreeding caused by small effective population size is therefore of great concern in e.g. conservation genetics given the risk of inbreeding depression and reductions in genetic variance (Allendorf et al., 2013; Crow and Kimura, 1970; Falconer and Mackay, 1996; Frankham et al., 2010; Kristensen and Sørensen, 2005; Lynch and Walsh, 1998). Several studies have shown that the genetic stress imposed by inbreeding is highly dependent on the environment. Inbreeding depression is particularly evident under stressful environmental conditions, suggesting an interaction between inbreeding and environmental stress (Armbruster and Reed, 2005; Bijlsma and Loeschcke, 2012; Fox and Reed, 2011; Reed et al., 2012).

The detrimental consequences of inbreeding have been reported in a wide variety of traits and in organisms ranging from model

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organisms (Latter and Mulley, 1995; White, 1972), livestock (Wiener et al., 1992) and wild populations (Crnokrak and Roff, 1999). Often it is found that inbreeding depression is more severe for life history traits (e.g. viability, longevity and fecundity) than for morphological, physiological or behavioral traits (DeRose and Roff, 1999; Frankham et al., 2010; Wright et al., 2008). Little is known about the effect of inbreeding and genetic drift on the overall metabolic rate of organisms. The metabolic rate of an organism is an important trait to consider as it sums its overall energetic turnover and thereby represents a measure of the costs of living which affects e.g. population growth rates and longevity (Hill et al., 2008; Parsons, 2005; Speakman, 2005). Myrand et al. (2002) and Tremblay et al. (1998) have argued that higher resistance to environmental stress in heterozygous blue mussels, *Mytilus edulis*, can be explained by their lower basic metabolic needs, leaving more energy available for resisting stressful conditions. Likewise, Ketola and Kotiaho (2009) showed that inbred crickets, *Gryllodes sigillatus*, had increased maintenance metabolism when compared to an outbred group. A similar result was found when comparing one inbred line of *Drosophila melanogaster* against its founder population (Lints and Lints, 1968). If homozygotes have higher metabolic rate, this suggests that the genetic stress imposed by inbreeding entails an energetic cost such that inbred individuals are more dependent on energy reserves to maintain *the same* level of physiological homeostasis. Thus, according to this hypothesis heterozygotes have a surplus of energy that can be used in situations when the organism experiences stressful conditions (Koehn and Bayne, 1989; Parsons, 2004, 2005).

The aim of this study is to investigate the effect of small population size on standard metabolic rate of a population of *D. melanogaster* at different temperatures. We examined the standard metabolic rate to investigate basic differences between inbred and outbred individuals. Other measures, such as aerobic scope, would require maximal performance of the flies, which is very difficult to obtain. We measured the metabolic rate at three different temperatures (low stressful, benign (corresponding to the temperature at which inbreeding took place) and high stressful). We developed a method that estimates the standard (least active) metabolic rate, with which we compared the mass corrected metabolic rate of inbred and outbred lines of *D. melanogaster*. Our hypothesis is that inbred *D. melanogaster* on average have a higher standard metabolic rate than outbred lines. This prediction is partly based on previous results showing that genes involved in major metabolic pathways, such as the fatty acid metabolism, are upregulated in inbred compared to outbred individuals of *Drosophila* (Garcia et al., 2012; Kristensen et al., 2006). Further, a major heat shock protein, Hsp70, has been shown to be upregulated in more homozygous individuals of *D. melanogaster* and *Drosophila buzzatii* (Kristensen et al., 2002; Pedersen et al., 2005). However, increased mRNA abundance may not lead to increases at the protein level (Feder and Walser, 2005; Suarez and Moyes, 2012). Interactions between inbreeding and the environment is often observed to affect fitness at the functional level (Reed et al., 2012). Also studies on gene expression have revealed inbreeding by temperature interactions at the biological level with inbreeding effects on major metabolic pathways being accentuated under high temperature stress in *D. melanogaster* (Kristensen et al., 2006). Therefore we further hypothesize that inbreeding and temperature stress interact resulting in a more pronounced difference in metabolic rate between inbred and outbred lines at the stressful temperatures. Finally, we hypothesized that genetic drift in the small populations lead to increased variance in metabolic rate between inbred lines compared to outbred lines. We used the lowest estimates of metabolic rate over a 24-h period to ensure that the best estimate of standard metabolic rate was achieved.

2. Materials and methods

2.1. Population and breeding regime

The mass-bred population of *D. melanogaster* was founded by 589 inseminated females caught in Odder (55°56′42.46″N, 10°12′45.31″E), Denmark, in October 2010. Each female contributed with ten virgin offspring (five males and five females) to the mass-bred population, which was maintained with an approximate population size of 6000 individuals for two generations (25 ± 1 °C, 12-h light/12-h dark cycles).

Inbreeding is expected to decrease the within line variation and increase variation between lines (Falconer and Mackay, 1996). On the contrary outbred lines maintained at large population sizes and with approximately random mating are expected to be more similar to each other (Falconer and Mackay, 1996). Therefore, fewer lines are needed to capture the genetic variance in outbred lines. From our mass-bred population we established ten inbred lines, each from five males and five females, and three outbred lines, each from 250 males and 250 females. The lines were maintained at these population sizes with equal sex ratios for 25 generations. Inbred and outbred lines were kept in vials containing 7 mL Leeds medium and in ten bottles with 35 mL Leeds medium, respectively. Leeds medium is a standard oatmeal-sugar-yeast-agar *Drosophila* medium. All lines were maintained in a temperature regime with a mean of 24.8 ± 0.5 °C. The temperature regime followed a Gaussian distribution, with a daily temperature peak at 27.5 ± 0.5 °C and night temperature minimum of 23.5 ± 0.5 °C. The light regime followed the same rhythm, with very low intensity during night, and a peak intensity coinciding with the temperature peak. In each generation, flies of age 5–8 days laid eggs in one short period (12 h) and one long period (24 h). After 3 days, the amount of food consumed by larvae in the two egg-laying periods was compared, and an egg-laying period was chosen for each line such that the larval density was equalized between lines. When all flies had emerged from the chosen egg-laying period, five females and five males or approximately 250 females and 250 males, depending on breeding regime, were randomly selected to establish the next generation. From generation 25 to 31, where the experiments were initiated (see Table 1), each line was distributed in three bottles containing 35 mL Leeds medium, with a total of 600 flies.

The expected level of inbreeding after t generations (F_t) was calculated from the equation $F_t = F_{t-1} + (1 - 2F_{t-1} + F_{t-2})/2N_e$ (Crow and Kimura, 1970), assuming $F_0 = 0$ and N (census size) = N_e (effective population size). After 25 generations F_t was expected to be ≈0.70 for the inbred lines. As it is likely that $N_e < N$, the estimated inbreeding coefficients represent the lower boundary of the realized inbreeding coefficient.

2.2. Experimental design

Flies used for measurement of metabolic rate were established from four consecutive generations maintained at standard conditions, 25 ± 1 °C, cycles of 12 h light 12 h dark (Table 1). Development of experimental flies was controlled for density by having approximately 40 eggs in each vial with 7 mL Leeds medium. 24 h after flies emerged non-virgin males were collected (assuming they had all mated within the first day of eclosion) (Eastwood and Burnet, 1977). From each of the ten inbred and three outbred lines, 18 replicate samples of 20 male flies were collected under light CO_2 anesthesia. These flies were placed in new vials with 4 mL of Leeds medium (25 ± 1 °C, cycles of 12 h light and 12 h dark in a temperature cabinet maintained at 50% RH) and transferred to fresh vials after two additional days. Experiments were performed on 5-day-old flies to ensure sufficient recovery time from CO_2

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Table 1
Information on the experimental temperatures, the generations of experimental flies used, number of replicates per generation per line, and the duration of closed and flush periods during measurements of CO_2-production. The duration (in seconds, s) of flush- and the closed-periods were varied across temperature to obtain low signal-to-noise ratio. At each temperature (with standard errors, SE) flies from different generations were used. We performed three extra measurements at the lowest temperature, which involved flies from a fourth generation.

Measured temperature (±SE)	Flush period (s)	Closed period (s)	Generation	Number of replicates
6.7 ± 0.04 °C	540	8100	31, 34	5, 3
26.1 ± 0.08 °C	180	2700	32	5
32.8 ± 0.03 °C	90	1350	33	5

anesthesia. There is no evidence of metabolic alterations caused by CO_2 anesthesia when flies have recovered more than 24 h after anesthesia (Colinet and Renault, 2012).

2.3. Metabolic rate and activity

Metabolic rate of *D. melanogaster* lines (inbred and outbred) was indirectly estimated by the rate of CO_2 production ($\dot{V}_{CO_2}$) (Glazier, 2005) using repeated measurements of stop-flow respirometry (Lighton and Halsey, 2011). Male flies from the different lines were randomly assigned to one of 16 cylindrical glass chambers (metabolic chambers, dimensions: *D*: 20 mm *L*: 70 mm, approximately 20 individuals in each chamber). Within each chamber flies had access to a piece of filter paper (15 mm × 15 mm) moistened with a 0.3 mL solution of 4% sugar and 2% agar to avoid starvation and desiccation. Each of the chambers was enclosed by an activity detector (AD2 Activity Detector, Sable Systems, Las Vegas, Nevada, USA), to allow a qualitative assessment of the flies' activity level in the chambers.

Two parallel 8-channels-multiplexers (RM Gas Flow Multiplexer, Sable Systems, Las Vegas, Nevada, USA) controlled the sequentially flushing and closing of the metabolic chambers such that the stop-flow respirometry system enabled us to obtain repeated measures of $\dot{V}_{CO_2}$ in 16 parallel metabolic chambers over 24 h. During the flush phase the metabolic chambers were perfused with CO_2 striped air (air passing a soda lime column (MERCK Millipore, Darmstadt, Germany) at a fixed rate of 150 mL min^{-1}). Airflow was controlled by an adjustable mass flow meter (Side-Trak®, Sierra Instruments, Monterey, California, USA) controlled by a flow controller (MFC 2-channel v. 1.0, Sable Systems, Las Vegas, Nevada, USA). After the flush phase the metabolic chamber was closed while the remaining 15 chambers were flushed sequentially in a similar manner such that the duration of the closed phase is 15 times the duration of the flush phase. The air leaving the metabolic chambers passed a calcium chloride column (Appli-Chem, Darmstadt, Germany) to remove water before entering a CO_2 analyzer (Li-6251 CO_2 Analyzer, Li-COR Environmental, Lincoln, Nebraska, USA).

Data were sampled at 1-s intervals using an UI2 interface (UI-2 Data Acquisition Interface, Sable Systems, Las Vegas, Nevada, USA) and collected using ExpeData software (ExpeData-UI2 software, Sable Systems, Las Vegas, Nevada, USA). Temperature within one of the 16 metabolic chambers was recorded using a data logger (iButton® Data Loggers, Maxim, Sunnyvale, California, USA) from which data were extracted by OneWireViewer (Maxim, Sunnyvale, California, USA). Measurements were performed independently at three experimental temperatures by placing the metabolic chambers in a climate chamber maintained at 6.7 ± 0.04 °C, 26.1 ± 0.08 °C or 32.8 ± 0.03 °C (24 h dark). The three experimental temperatures were chosen to represent: a benign temperature (26.1 °C) similar to the developmental temperature; a high stressful temperature known to induce a nonlethal stress response (Hoffmann et al., 2003); and a low temperature known to induce a nonlethal stress response (Colinet et al., 2010). The low temperature did also ensure measurements at which locomotor activity is rarely observed.

13 Experimental lines and three empty control chambers were included in all runs. As a consequence of different experimental temperatures the flush and closed periods were varied to obtain an appropriate signal to noise ratio (CO_2 production rates are reduced at lower temperatures). Thus periods were longer when measurements were run at low experimental temperature (Table 1). As a consequence of the longer closing and flushing periods fewer repeated measures were obtained at low temperature (10 independent measurements per day) than at benign (30 measurements) and high temperatures (60 measurements). Spontaneous activity is diminished at the low temperature therefore fewer measurements are needed to obtain periods with inactivity. On the contrary, the relative shorter period at high temperature increases the accuracy of identifying periods with low activity.

Activity of flies in a metabolic chamber was only measured when the metabolic chamber was flushed. The AD2 activity detector emits a beam of infrared light (880 nm), which is reflected on the surface of the activity chamber and the light intensity is then recorded by a photodetector sensitive to light at 880 nm. The intensity of the light detected by the photoreceptor depends on the degree of interruption and deflection caused by the movements of the flies within the chamber. Thus, locomotor activity is measured as the changes in light intensity in the form of positive or negative deflections of signal (Sable Systems, 2009). Activity intensity was estimated as the cumulative sum of absolute differences in deflection (ADS) between consecutive data points (Lighton and Turner, 2004). The activity was measured during flushing of CO_2 whereas $\dot{V}_{CO_2}$ is the CO_2 produced during the closed period. Thus, there is a time lag between the two measurements. To compensate for this, the activity level for a given closed period was estimated as the average activity of the recordings immediately before and after the closed period. The activity measurements are only semi-quantitative measures of locomotor activity and comparative analyses of activity between the metabolic chambers are not possible. However, activity measurements are included to validate our estimate of standard metabolic rate since it enables identification of periods with least activity in a chamber (see Fig. 1C).

2.3.1. Analysis of respirometry data

Raw data from the metabolic rate measurements were processed by a script in Mathematica (version 7.0, Wolfram Research, Champaign, Illinois, USA), which identified the start, found the baseline value of each CO_2 peak and integrated the area between the graph and the baseline (Fig. 1B). Signals from all measurements were manually examined, and measurements which deviated from a standard CO_2-trace (Fig. 1B) were discarded.

The experimental protocol rendered multiple measurements of the same batch of flies over a 24-h period. Due to handling stress and CO_2 contamination acquired during preparation of the chambers, the first rounds (6.7 °C: 2 rounds, 26.1 °C: 4 rounds or 32.8 °C: 6 rounds) of recordings were discarded. To correct for any instrumental leakage and/or CO_2 diffusion over the tubing the signal from the corresponding empty chambers was subtracted from each recording (Fig. 1A). CO_2 production was expressed relatively to dry body mass and corrected to Standard Temperature and Pressure Dry (STPD) conditions. In addition, a correction of $\dot{V}_{CO_2}$ was performed to correct for differences in body mass and thereby achieve $\dot{V}_{CO_2}$ of flies of same body mass, 0.26 mg (mean dry mass of all flies) dry mass pr. individual according to:

$$\frac{m}{w} = aW^{(b-1)} \iff \frac{m}{w}(y) = \frac{m}{w}(x) \cdot \left(\frac{w(y)}{w(x)}\right)^{-0.25},$$

(Hill et al., 2008; Schmidt-Nielsen, 1995)

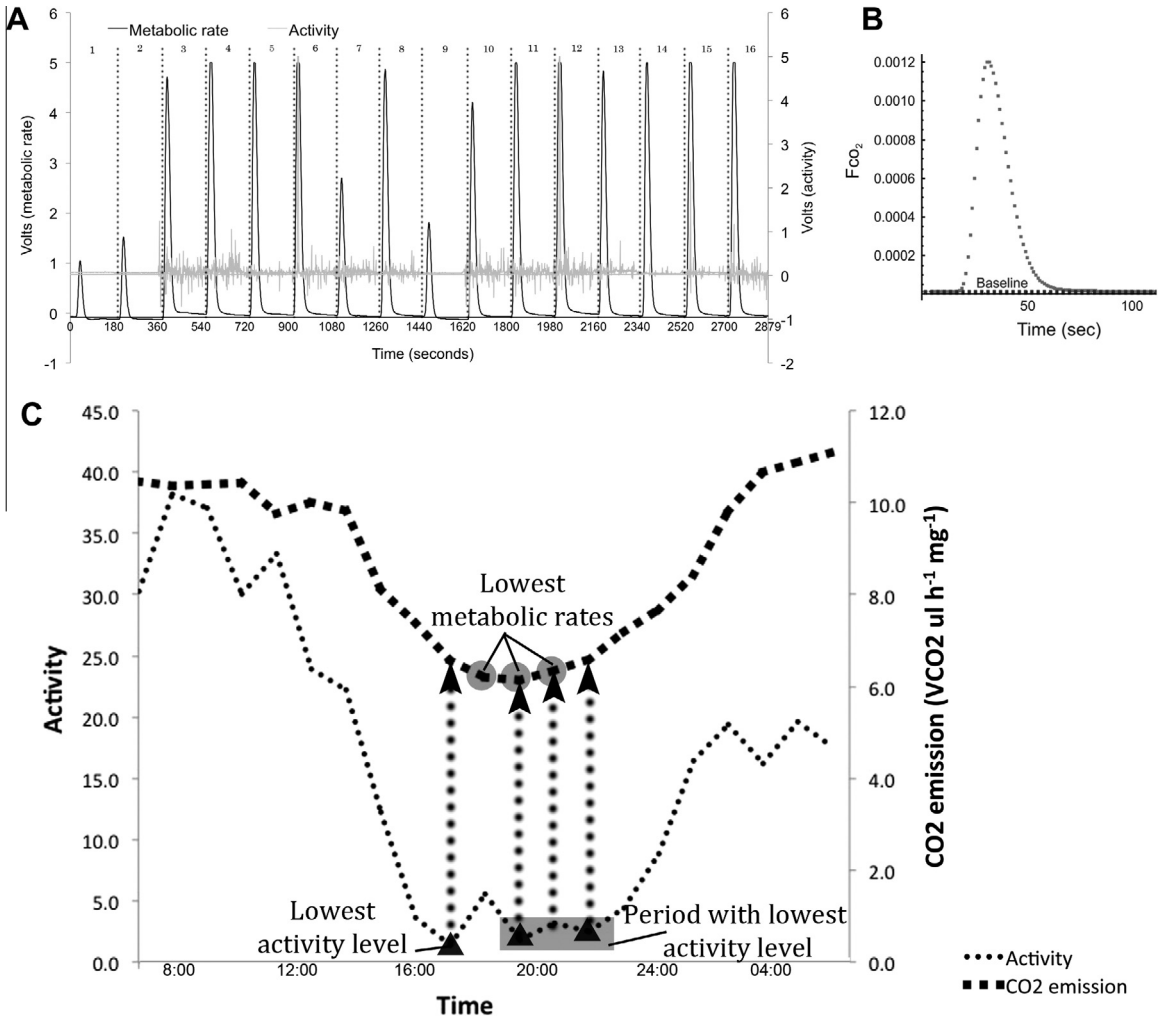

Fig. 1. Measurements of activity and metabolic rate (approximation from CO_2 emission ($\dot{V}_{CO_2}$), μL h^{-1} mg^{-1}) at 26.1 °C. (A) 16 Measurements of metabolic rate and activity, with three empty metabolic chambers (1, 2 and 9). (B) $\dot{V}_{CO_2}$ curve of produced CO_2 from the open and closed periods. (C) Activity and metabolic rate measurements plotted against time of measurement. Topmost dashed graph: metabolic rate, lowermost dashed graph; activity measurement. Three methods for estimating SMR: shaded circles: method using the three lowest metabolic rate measurements, grey triangles: method finding the three lowest activity measurements and using the corresponding metabolic rates, shaded square: method identifying the three lowest consecutive activity measurements and the corresponding metabolic rates.

where m/w is the mass specific metabolic rate, w is the dry mass, b is the scaling coefficient, x and y are the body mass of the measured and "standard" individuals, respectively. Here we assume an interspecific scaling coefficient of $b = 0.75$ (Schmidt-Nielsen, 1995). The value of the scaling coefficient is a controversial topic (see e.g. Glazier, 2005) and several factors are proposed to influence it, e.g. life stage and potentially inbreeding. Data were standardized by the mean metabolic rate of each day to diminish any day effects. By doing this we obtained the same mean on each measurement day.

2.3.2. Estimate of standard metabolic rate

To reduce the confounding effects caused by activity, diurnal rhythm and handling stress, three separate approaches were initially used to identify appropriate data points to estimate standard metabolic rate (SMR). The first method estimates SMR using the average of the three lowest measures of $\dot{V}_{CO_2}$ during one day of recordings (Fig. 1C); the second method identified the three lowest activity measurements during the day and used the corresponding metabolic rates from these time points; and the third method identified the three lowest consecutive activity measurements and used the corresponding metabolic rates (Fig. 1C).

2.4. Body mass

After one day of respirometry measurements the flies were transferred to 1.5 mL Eppendorf tubes, and stored in a −80 °C freezer. The wet mass of the flies was determined (Sartorius Laboratory Balance, type 1712, 6 decimal accuracy, Göttingen, Germany) after defrosting, and the dry mass was measured after drying for 24 h at 60 °C. Water content was calculated as mg H_2O/mg dry mass.

2.5. Statistical analyses

To compare the three different approaches used to select data points to obtain the best estimate of standard metabolic rate we conducted a pairwise comparison with Spearman's Rank correlation to test the linear association between the three different approaches (Whitlock and Schluter, 2009).

To investigate whether breeding regime had an effect on standard metabolic rate we analyzed the data using linear mixed models. Using model selection we sequentially reduce models removing variables and testing the effect using likelihood ratio tests; $\Lambda = 2 \cdot (l_a - l_b)$, where l_a and l_b are the log-likelihood functions

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for the two nested models, *a* and *b*. The distribution of the test statistic (Λ) may deviate from a χ^2-distribution because of the random components in the mixed models. Therefore we used parametric bootstrapping generating an empirical distribution of Λ under the reduced model and compared this to the observed Λ. 10.000 empirical Λ were obtained and the empirical *p*-value for a one-sided test is equal to the proportion of empirical Λ that are larger than the observed Λ using a cutoff level at 5%.

The starting model is $y = B^*C + L + D + RS$, where *y* is the mass corrected standard metabolic rate (non day-corrected data were used in the models), *B* is breeding regime (inbred vs. outbred), *C* is temperature, *L* is line, *D* is day of measurement and *RS* is random slope (Schielzeth and Forstmeier, 2008) between temperature and line. *B* and *C* are treated as fixed effects and *L* and *D* as random effects, with lines nested within breeding regime. The effect of breeding regime on dry mass and water content was likewise analyzed with linear mixed models: $y = B + L + D$, with *y* being dry mass or water content, using the approach described above.

All phenotypic data were checked for outlying data points, and model assumptions of normality- and homoscedasticity of the residuals were assessed. To fulfill assumptions the metabolic rate data were subsequently log-transformed. Data on dry mass- and water content were not transformed. Data points where the residuals were extreme were examined for possible measuring errors. The statistical analyses were performed with and without possible outlying data points to investigate the effect of the extreme values. Removing data points was done with caution and directional change of conclusions was dissuaded. After careful examination, only three points were removed due to measurements errors, all from the water content dataset.

All the statistical analyses were conducted in *R* (R Core Team, 2013) and the linear mixed models with the *R* package *lme4* (Bates et al., 2013).

3. Results

The effect of breeding regime on the body mass of *D. melanogaster* was marginal non-significant at 5% ($p = 0.052$, Fig. 2A). On average the dry mass of inbred lines (0.259 ± 0.006 mg) was 9% lower than in outbred lines (0.285 ± 0.003 mg). No significant difference in water content between breeding regimes ($p = 0.23$) was observed (Fig. 2B). Inbred lines contained on average 2.118 ± 0.009 mg $H_2O\cdots(\text{mg dry mass})^{-1}$ whereas outbred lines contained 2.032 ± 0.002 mg $H_2O\cdots(\text{mg dry mass})^{-1}$ which corresponds to 68% and 67% water in an inbred and outbred fly.

To obtain the best estimate of standard metabolic rate we initially used three different approaches for selection of appropriate data points from the measurements (Fig. 1C). The overall pattern of standard metabolic rate was the same, independent of which of the three approaches were used (Fig. 3A–C). This is also evident from the similarity seen in the pairwise correlation between data obtained from these approaches (Fig. 3D, $\rho = 1$, $p = 0$). Given that the standard metabolic rate was expected to result in the lowest $\dot{V}_{CO_2}$ we chose to use the approach that calculates the mean of the three lowest $\dot{V}_{CO_2}$ over the 24-h of measurements (Figs. 1C and 4).

We did not find any evidence of a significant interaction between temperature and breeding regime ($p = 0.56$, Table 2) nor did we find evidence for a significant effect of breeding regime ($p = 0.20$, Table 2) alone. Both temperature (*C*) and the random line effect (*L*) did significantly affect the models ($p < 0.001$ and $p = 0.011$, respectively, Table 2). Next, we investigated whether there were differences among the three outbred lines at each temperature (comparing model 5 with model 7, Table 2). No evidence against equality among the outbred lines within each temperature could be found ($p = 0.96$, Table 2), however there were indeed differences between the combined outbred population and the inbred lines ($p = 0.0024$, Table 2), which supports the hypothesis of increased variance in metabolic rate of inbred lines (Fig. 4). At the low temperature the mean metabolic rate of inbred *D. melanogaster* was 1.28 ± 0.043 μL CO_2 h^{-1} mg^{-1} dry mass compared to outbred individuals with a mean metabolic rate of 1.18 ± 0.015 μL CO_2 h^{-1} mg^{-1} dry mass. At the high temperature the mean metabolic rate was measured to 11.11 ± 0.37 and 11.48 ± 0.53 μL CO_2 h^{-1} mg^{-1} dry mass for inbred and outbred individuals, respectively. Inbred *D. melanogaster* at benign temperature had a mean metabolic rate of 8.50 ± 0.64 μL CO_2 h^{-1} mg^{-1} dry mass compared to 7.13 ± 0.36 μL CO_2 h^{-1} mg^{-1} dry mass for outbred individuals (Table 3).

To validate our results we first investigated the effect of the scaling component (see Section 2.3.1), second our statistical model (see Section 2.5) by applying an alternative model, and third using across day average instead of average of minimum values (see Section 2.3.2). By varying the scaling component within possible values, from 0.67 to 1 (Glazier, 2005), our overall conclusions do not change with no indication of a directional effect of inbreeding (data not shown). An alternative statistical approach is to use non-mass specific metabolic rate (μL CO_2 h^{-1}) and total fly mass per metabolic chamber and the average mass per line as covariates and otherwise do as in the previous analysis (data not shown). The results from this analysis are in agreement with our main results. Finally, we conducted the analyses with a day-average of the CO_2 emission and these results are also in concordance with our main findings (data not shown).

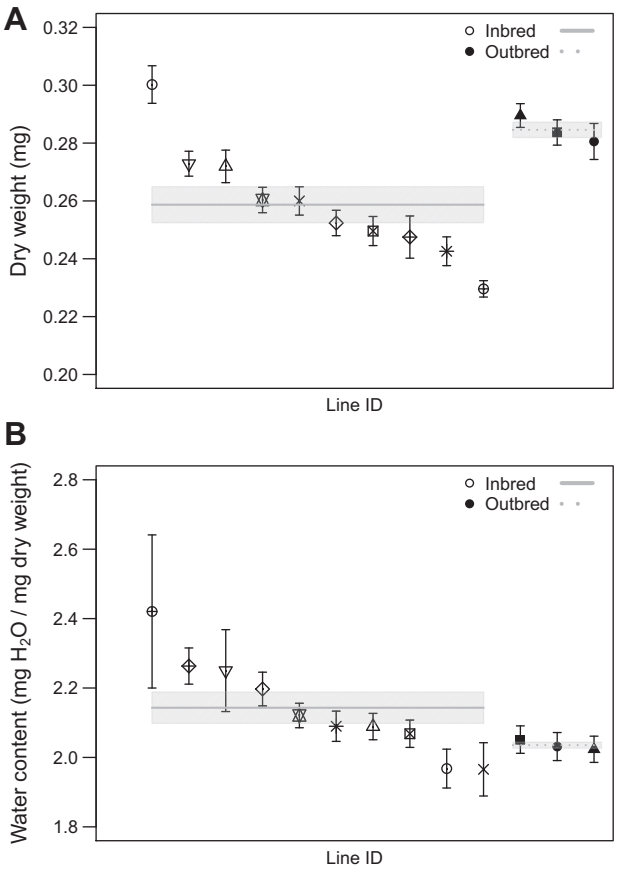

Fig. 2. Line specific mean dry mass (A) and mean water content (B) of inbred (open symbols) and outbred (closed symbols) lines of *D. melanogaster* sorted by decreasing values. The symbols are line specific and correspond to those in Fig. 4. Error bars represent the standard error of means across days. Horizontal grey lines (full = inbred, dotted = outbred) represent the mean within breeding regime, and the shaded areas are the standard errors across lines.

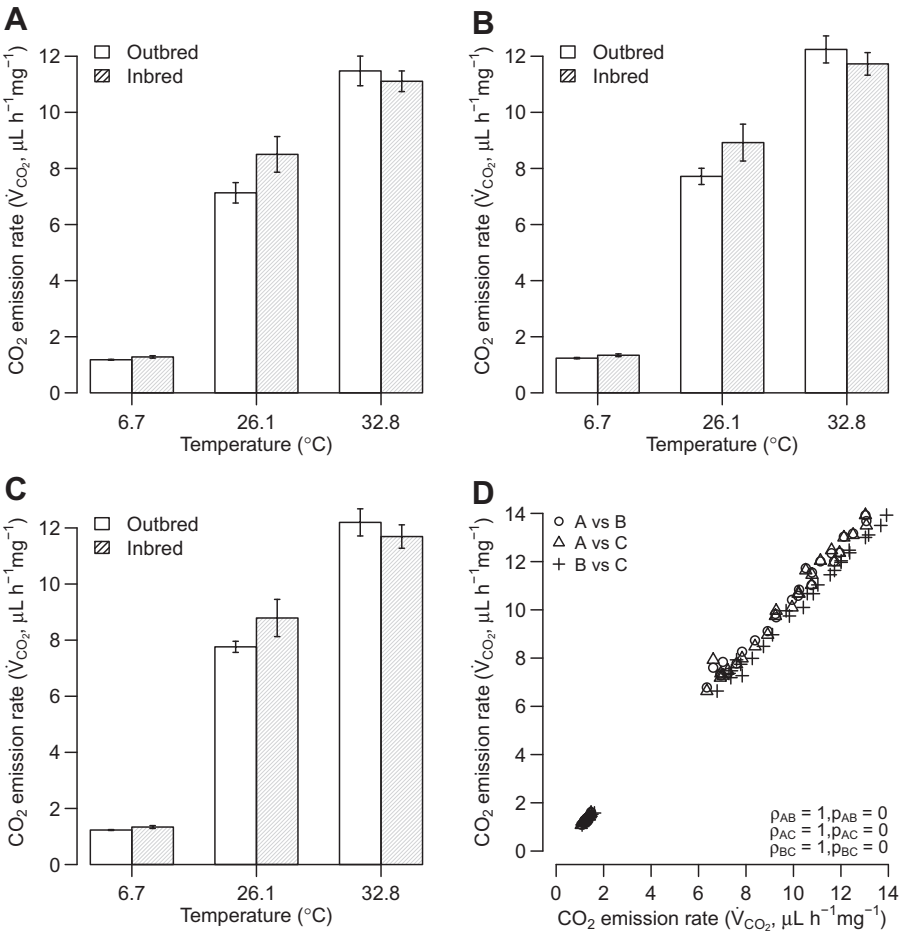

Fig. 3. (A–C) Overview of the level of standard metabolic rate (approximation from CO_2 emission ($\dot{V}_{CO_2}$), µL h^{-1} mg^{-1}) estimated by three different approaches. Standard metabolic rates within breeding regimes and error bars indicate the standard error across lines. (A) Approach using the three lowest measurements of metabolic rate in a period of 24 h. (B) Approach identifying the three lowest records of locomotor activity in a period of 24 h and using the time corresponding metabolic rate measurements. (C) Approach finding the three lowest consecutive locomotor activity levels in a period of 24 h and using the time corresponding metabolic rate records. (D) Pairwise correlation (Spearman's rank correlation coefficient, ρ) between the three different methods, ρ_{AB}, ρ_{AC} and ρ_{BC} with corresponding p-values. Data points are line means obtained from the three different approaches.

4. Discussion

In the present study we measured the mass corrected metabolic rate in a small ectothermic animal, *D. melanogaster*, using stop-flow respirometry over a 24-h period with repeated measurements. We hypothesized an energetic cost associated with inbreeding resulting in a higher metabolic rate of inbred individuals compared to outbred individuals. We hypothesized that this effect will be more pronounced under stress, as a consequence of an interaction between intrinsic and extrinsic stress. Further we hypothesized that the variance in metabolic rate between inbred lines was higher compared to the observed variance between control lines. By attempting to control for factors that could potentially bias our estimates of metabolic rate, such as temperature, body mass and locomotor activity, we were able to collect evidence to test these hypotheses.

Precision of measurements of standard metabolic rate is dependent on the method applied. The three main factors decisive for the metabolic rate of animals are temperature, locomotor activity and body mass (Hill et al., 2008). Using the current method and approach of obtaining estimates of standard metabolic rate we were able to partly control for these factors. We have compiled a set of estimates from the literature (Fig. 5), which contains data from both inbred and outbred *D. melanogaster* and from measurements using both estimates of $\dot{V}_{CO_2}$ and $\dot{V}_{CO_2}$ which we converted to the same units (µL CO_2 h^{-1} mg^{-1} dry mass, see legend Fig. 5). Comparison of our estimates with the literature values shows that our estimates of metabolic rate in general are lower, with two exceptions (Fig. 5, reference point 10 (Promislow and Haselkorn, 2002) and reference point 12 (Ueno et al., 2012)). The estimate from Ueno et al. (2012) is very close to ours, but the estimate from Promislow and Haselkorn (2002) is considerable lower than ours. Their distinct lower values may be attributed to their use of CO_2 anaesthetization 15 min prior to measurements. It is known that CO_2 exposure will cause metabolic changes up to 14 h after exposure, and it has been reported that some metabolites are downregulated in consequence of CO_2 exposure (Colinet and Renault, 2012). Moreover, the oxidative phosphorylation and other enzymes are inhibited and impaired under and after CO_2 anaesthetization, respectively (Colinet and Renault, 2012). These factors are likely to explain the low values from the study of Promislow and Haselkorn (2002). None of the studies in our comparison have accounted for locomotor activity, however five did monitor the activity (Berrigan and Hoang, 1999; Berrigan and Partridge, 1997; Lighton and Schilman, 2007; Schilman et al., 2011; Ueno et al., 2012). Locomotor activity may explain why these many published values are higher than in our study. Contributing to the differences in the estimates are also differences in the experimental design, such as

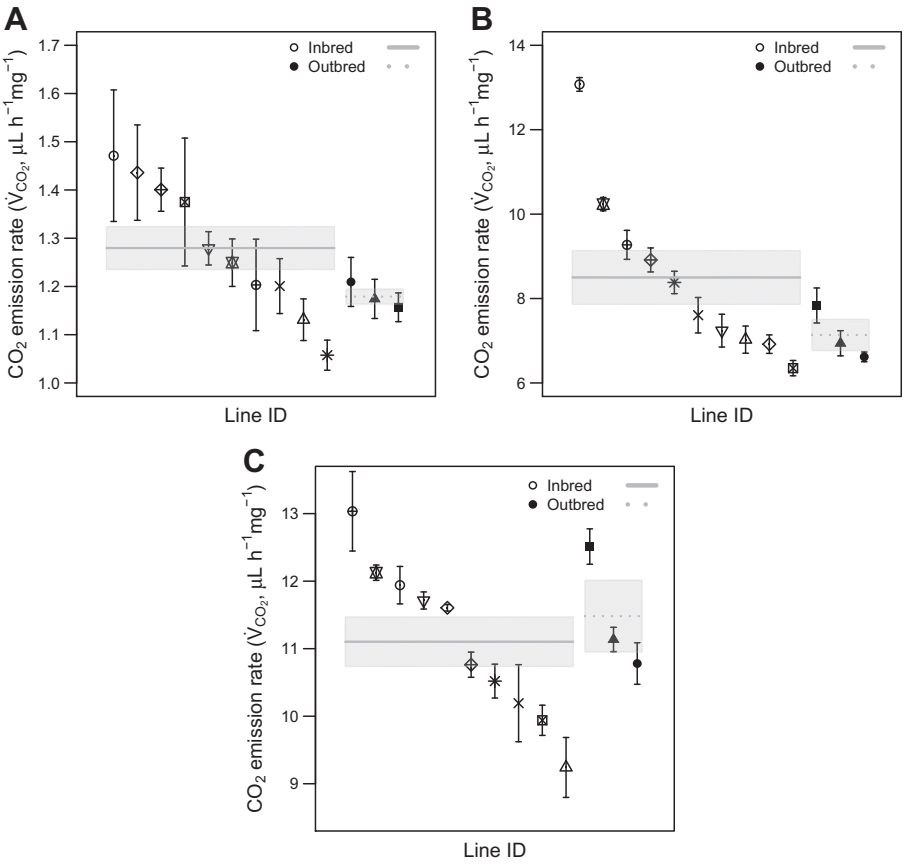

Fig. 4. Line specific standard metabolic rates (approximation from CO_2 emission ($\dot{V}_{CO_2}$), µL h^{-1} mg^{-1}) of inbred (open symbols) and outbred (closed symbols) lines at the three experimental temperatures, (A) 6.7 °C, (B) 26.1 °C and (C) 32.8 °C. The symbols are line specific and correspond to those in Fig. 2. Error bars show the standard error of means across days. Horizontal grey lines (full = inbred, dotted = outbred) represent the mean standard metabolic rate within breeding regime, and the shaded areas are the standard errors across lines.

Table 2
Showing sequential model selection, where variables one at a time are removed starting with the full model ($B^*C + L + D + RS$). Pr > Λ is a one-sided test equal to the proportion of the empirical test statistics (Λ) that are larger than the observed Λ using a cutoff level at 5%. If the reduction is not significant (Pr > 5%) there is no evidence against the reduction, thus the reduction to the model with fewer parameters is performed.

Nr.	Models	Pr > Λ
1	$B^*C + L + D + RS$	
2	$B^*C + L + D$	<0.001
3	$B + C + L + D + RS$	0.56
4	$B + L + D + RS$	<0.001
5	$C + L + D + RS$	0.20
6	$C + D + RS$	0.011
7	$C + L1 + D + RS$	0.96
8	$C + D + RS$	0.0024

B is breeding regime (inbred *vs.* outbred), *C* is the temperature, *L* is random line effect, *D* is a random day component and *RS* is random slope, and *L1* is a new line component with 1 group of outbred lines (the three outbred lines merged into one variable).

Table 3
Average metabolic rate (µL $CO_2 \cdot h^{-1}$ mg^{-1} dry mass) of inbred and outbred *D. melanogaster* at the three experimental temperatures. SE indicates the standard error.

Temperature	Breeding regime	Mean metabolic rate (µL $CO_2 \cdot h^{-1}$ mg^{-1} dry mass)	SE
6.7 °C	Inbred	1.28	0.043
–	Outbred	1.18	0.015
26.1 °C	Inbred	8.50	0.64
–	Outbred	7.13	0.36
32.8 °C	Inbred	11.11	0.37
–	Outbred	11.48	0.53

whether the estimates are based on individual or group measurements. However, in *Drosophila* studies no such differences between individual- and group records have been found (Van Voorhies et al., 2004). Whether to measure O_2 consumption, CO_2 production or both might also contribute to differences observed between studies. Especially for small animals with very low metabolic rate, measuring CO_2 production is easier. Detecting very small gas differences are more accurate from a zero baseline, than a small decrease from the surrounding air with high amount (Lighton and Halsey, 2011).

Reliable estimates of standard metabolic rate are highly dependent on several factors, as shown above. The low estimates obtained in our study suggest that we have been efficient in controlling for the confounding factors. Thus we have confidence that we report the metabolic rate of resting animals. Moreover, we have shown that our results are robust given that altering factors, e.g. the scaling factor, did not change the results, which is in agreement with a recent study were it was shown that inbreeding did not affect the scaling component in male *D. montana* (Ketola et al., 2013).

In natural populations body size may influence traits associated with survival, mating systems and other life history traits such as number and size of offspring (Freeman and Herron, 2007; Santos et al., 1988; Singh et al., 2002). Reduction in body size may be a result of reduced cell size and/or cell number (Arendt, 2007;

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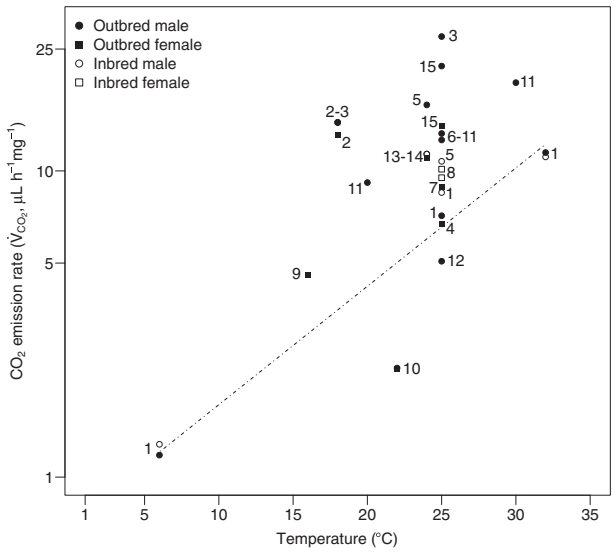

Fig. 5. Estimates of standard metabolic rate from the present study and from representative published studies. The list encompasses fourteen representative studies of measurements of metabolic rate from male and female *D. melanogaster*. The list contains data from both inbred (open symbols) and outbred (closed symbols) lines and from measurements using both estimates of $\dot{V}_{CO_2}$ and $\dot{V}_{CO_2}$. All data have been converted to µL CO_2 h^{-1} mg^{-1} dry weight. In studies where the values of metabolic rate not are provided in tables or text, we estimated the values by graphical interpretation. In cases where water content is not reported, we assumed to be the same as in our study (68%). If RQ values were not reported we assumed a RQ = 1 (Berrigan and Partridge, 1997). Dashed line is an exponential fit of standard metabolic rate (R^2 = 0.9959, p = 0.04) with our outbred data. Numbers next to the data points refer to the different studies: (1) Present study, (2) Berrigan and Hoang (1999), (3) Berrigan and Partridge (1997), (4) Djawdan et al. (1997), (5) Gibbs and Markow (2001) (6) Lighton and Schilman (2007), (7) Lints and Lints (1968), (8) Mikkelsen et al. (2010), (9) Overgaard et al. (2010), (10) Promislow and Haselkorn (2002), (11) Schilman et al. (2011), (12) Ueno et al. (2012), (13) Van Voorhies et al. (2003), (14) Van Voorhies et al. (2004) and (15) Wit et al. (2013).

Vijendravarma et al., 2011). This may also contribute to biochemical differences and thereby affect metabolic rate. As shown here, inbreeding decreased the body mass of *D. melanogaster* (Fig. 2A) by 9% compared to outbred lines.

The main hypothesis tested in the present study was that inbreeding results in a higher standard metabolic rate of *D. melanogaster*. Increased standard metabolic rate in inbred individuals was hypothesized being a consequence of an upregulation of gene expression in general in inbred *D. melanogaster* (Garcia et al., 2012), but in particular an increase in the cellular stress response system in homozygous individuals (Ayroles et al., 2009; Garcia et al., 2013; Kristensen et al., 2010). According to this idea higher metabolic rate of inbred lines could be expected at all three temperatures. Our results did not confirm this hypothesis since the effect of breeding regime was not significant (Fig. 4, Table 2). Previous studies have suggested higher metabolic rate in inbred populations (Ketola and Kotiaho, 2009; Lints and Lints, 1968; Myrand et al., 2002, but see Mikkelsen et al. (2010)). There seem to be a tendency in the same direction at benign temperature (26.1 °C) where the standard metabolic rate of inbred lines was on average 19% higher than in outbred lines. However, the results from the statistical model led us to conclude that inbreeding effects on metabolic rate under the conditions tested in this experiment are minor and not of statistical significance (Fig. 4, Table 2).

Earlier attempts to assess the effect of inbreeding on metabolic rate of *D. melanogaster* have reported equivocal results (Lints and Lints, 1968; Mikkelsen et al., 2010). Lints and Lints (1968) reported discrepancies in metabolic rate between wild type and inbred *D. melanogaster*, whereas Mikkelsen et al. (2010) rejected that inbreeding affects the metabolic rate measured at benign temperature. However, several aspects differ between the study of Mikkelsen et al. (2010) and our present study. First, our level of inbreeding is substantial higher than that in Mikkelsen et al. (2010) ($F \approx 0.70$ *vs.* $F \approx 0.25$) and second, Mikkelsen et al. (2010) used a closed-system respirometry and measured O_2 consumption without accounting for activity. The main distinction between stop-flow- and closed-system respirometry is that the former allows for repeated measurements across a user-defined timescale, whereas the latter measures the produced/consumed gas for a short period (e.g. 1 h, as in Mikkelsen et al. (2010)). Despite these differences between the current study and the study by Mikkelsen et al. (2010) we reach the same conclusion, namely that inbreeding effects on metabolic rate in *D. melanogaster* are minor.

Organisms have evolved a range of mechanisms to survive stressful environmental conditions, including expression of stress proteins, changes in the metabolism and hormone concentrations (Hoffmann and Parsons, 1991; Hoffmann et al., 2003; Sørensen et al., 2003). These responses may also be activated by intrinsic stress factors, such as inbreeding (Ayroles et al., 2009; Cheng et al., 2006; Kristensen et al., 2002; Leimu et al., 2012; Pedersen et al., 2005). Accordingly, inbred individuals have been shown to express increased levels of stress proteins in situations that outbred individuals perceive as benign. For example, Kristensen et al. (2002) found a higher heat shock protein 70 (Hsp70) expression in inbred *D. buzzatii* and *D. melanogaster* lines at benign temperatures. Our hypothesis was that increased Hsp70 expression and expression of stress proteins in general (Dong et al., 2008; Roberts et al., 1997; Sørensen et al., 2003), could indirectly explain elevated standard metabolic rate of inbred lines at benign temperatures. Similarly, an upregulated expression of genes involved in metabolic pathways in inbred compared to outbred lines of *D. melanogaster* has been identified (Ayroles et al., 2009; Garcia et al., 2012; Kristensen et al., 2005, 2006). Moreover, Kristensen et al. (2006) found that genes and pathways involved in cellular stress responses and metabolic pathways, were upregulated in inbred *D. melanogaster* compared to outbred individuals and more so at high stressful temperatures. This inbreeding by temperature interaction observed at the mRNA level in Kristensen et al. (2006) led us to propose that an increased metabolic rate in inbred *D. melanogaster* would be even more exaggerated at stressful temperatures. This was clearly not observed (Fig. 4, Table 2). One explanation may be that the intrinsic stress induced by inbreeding reduces the capacity to respond plastically to an environmental stress such as elevated temperature. This phenomenon can be termed inbreeding depression for plasticity. An example is provided by Auld and Relyea (2010) showing that inbreeding caused reduced adaptive plasticity in predator induced shell thickness.

The last hypothesis that we put forward to test was that inbreeding increases the between line variance in metabolic rate. This hypothesis was accepted (Fig. 4, Table 2). This illustrates that (1) there is genetic variation for inbreeding effects on metabolic rate in the investigated population, and (2) line specific allele and genotype frequencies in individual inbred lines have strong effects on metabolic rate. Thus although the general effect of inbreeding on metabolic rate is not strong it seems as if the metabolic rate in some inbred lines is strongly impacted by inbreeding (Fig. 4). In future studies with a larger number of inbred lines it would be interesting to associate variation in metabolic rate between inbred lines with variation in other physiological and functional traits. Such research is needed to clarify what the actual energetic costs of inbreeding are when assessed through metabolic rates and will aid in clarifying the physiological basis of the commonly observed line specificity of inbreeding effects on performance.

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