doi:10.1111/evo.14126

Quantifying selection on standard metabolic rate and body mass in *Drosophila melanogaster*

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Received June 23, 2020
Accepted October 25, 2020

Standard metabolic rate (SMR), defined as the minimal energy expenditure required for self-maintenance, is a key physiological trait. Few studies have estimated its relationship with fitness, most notably in insects. This is presumably due to the difficulty of measuring SMR in a large number of very small individuals. Using high-throughput flow-through respirometry and a *Drosophila melanogaster* laboratory population adapted to a life cycle that facilitates fitness measures, we quantified SMR, body mass, and fitness in 515 female and 522 male adults. We used a novel multivariate approach to estimate linear and nonlinear selection differentials and gradients from the variance-covariance matrix of fitness, SMR, and body mass, allowing traits specific covariates to be accommodated within a single model. In males, linear selection differentials for mass and SMR were positive and individually significant. Selection gradients were also positive but, despite substantial sample sizes, were nonsignificant due to increased uncertainty given strong SMR-mass collinearity. In females, only nonlinear selection was detected and it appeared to act primarily on body size, although the individual gradients were again nonsignificant. Selection did not differ significantly between sexes although differences in the fitness surfaces suggest sex-specific selection as an important topic for further study.

KEY WORDS: **Basal metabolic rate, lifetime reproductive success, linear and nonlinear selection, multivariate selection, selection gradient, sexual dimorphism.**

Metabolic rate reflects the amount of energy that an organism needs to grow, reproduce, and survive. Because resources are limited, organisms must allocate their finite energy to competing demands, which forces allocation trade-offs that ultimately play an important role in shaping life-history strategies. All else being equal, energy allocated to self-maintenance cannot be invested in other energetic demands such as reproduction. However, reproducing at a high rate may necessitate a large metabolic machinery that translates into high-maintenance costs. As such, maintenance metabolism is likely to be linked to fitness (Burton et al. 2011), but studies so far have produced inconsistent results (Pettersen et al. 2018) and we therefore lack a good understanding of how selection shapes maintenance metabolism. This is perhaps not surprising given that estimating selection involves challenges such as measuring fitness and maintenance metabolism appropriately in a large number of individuals and parsing the relative contribution of highly collinear variables (e.g., body mass and metabolism) to fitness.

Quantifying fitness is technically challenging yet of utmost importance when studying selection. Lifetime reproductive success of an individual (total number of offspring produced) can be broken down into three main components: survival, fecundity, and reproductive success (pre- and postcopulatory). These components of fitness can vary independently and may relate differently to metabolic rate (Pettersen et al. 2018). For example, a high-maintenance metabolism may be beneficial to survival, but uses energy that otherwise could be invested in reproduction. Most estimates of selection on maintenance metabolism have, at best, quantified a portion of a single-fitness component such as overwinter survival (Jackson et al. 2001; Artacho and Nespolo

2009; Boratyński et al. 2010; Larivée et al. 2010; Careau et al. 2013; Zub et al. 2014) or output from a single reproductive event (Earle and Lavigne 1990; Stephenson and Racey 1993; Johnston et al. 2007; Hayes et al. 2009; Boratyński and Koteja 2010; Schimpf et al. 2012; Mariette et al. 2015). A small number of studies have attempted to relate metabolic rate to a more comprehensive measure of fitness (Blackmer et al. 2005; Pettersen et al. 2016), but we have limited insight into how total selection acts on this fundamental trait.

Measuring maintenance metabolism can also be challenging as, by definition, it excludes contributions due to activity, growth, and reproduction (Hulbert and Else 2004; Careau et al. 2015). In ectotherms, the "minimum cost of living" is measured as the standard metabolic rate (SMR): the metabolic rate of a resting, postabsorptive, and nonreproductive adult at a specified temperature. Meeting these criteria requires careful methodological considerations and can take time because individuals must be monitored over a sufficient period such that they relax and rest within the confinement of a metabolic chamber. Therefore, the criteria to measure SMR can impose major constrains on achieving sufficient sample sizes to estimate selection with precision. Small insects offer advantages as it is relatively easy to obtain to a large number of individuals, but their low metabolic rate makes it difficult to measure SMR precisely.

An additional challenge in estimating selection on metabolic rate is its strong (positive) collinearity with body mass (White 2011; White and Kearney 2013). Such collinearity can make it difficult to parse the relative strength of selection between these two traits. Collinearity can be alleviated by excluding traits that are not of interest, or by working with principal components (Zuur et al. 2010; Dormann et al. 2013; Chong et al. 2018; Harrison et al. 2019), but such approaches are not particularly useful when all of the correlated traits are of interest (e.g., metabolic rate and body mass are both hypothesized to be under selection). Historically, selection is estimated on SMR after correcting for body mass, usually by taking the residuals of a linear regression of SMR as function of mass (or by dividing SMR by body mass). However, this approach removes variation in SMR due to body mass and it is therefore not possible to estimate selection on the shared variation, nor does it allow correlational selection to be estimated for these traits. A preferable approach is to apply the Lande and Arnold (1983) framework to simultaneously quantify linear and nonlinear selection on both SMR, body mass, and their interaction. The Lande and Arnold (1983) framework is usually done by fitting a multiple linear regression with relative fitness as the response variable and the traits of interest (and their squared terms and second-order interactions for nonlinear selection) as predictors. When doing so, however, it is difficult to account for various nuisance parameters or other covariates that only apply to a subset of the traits without "doing statistics on statistics" (i.e., using residuals from a regression of a trait on its covariates). Such a two-step approach fails to carry forward uncertainty in estimates and can produce statistical artifacts (Garcia-Berthou 2001; Freckleton 2002; Morrissey 2014). A solution to this challenge is to use a multivariate approach to model the variance-covariance matrix between fitness, SMR, and body mass while correcting one or more traits for their unique covariates (in the current case for nuisance parameters unique to the estimation of SMR and relative fitness). Standard selection differentials and gradients can then be obtained from the residual covariance matrix (see Methods).

Here, our primary goal is to quantify multivariate selection on SMR and body mass. To do so, we build on the Lande and Arnold (1983) framework, employing multivariate mixed models to better account for trait-specific covariates. In measuring selection on these traits, we take advantage of a high-throughput respirometry system and a laboratory population of *Drosophila melanogaster* that has been evolving under a life cycle that facilitates a comprehensive measure of fitness. In this population, newly emerged adult flies interact for four days in a mating environment at a specific (and fairly low) density, after which females lay eggs for 24 hours to produce the next generation. Male fitness is therefore the number of offspring they sire during this four-day period, and female fitness is the number of adult offspring they produce during the 24-hour window. Our fitness measure therefore includes survival over these four days, fecundity, and reproductive success of the adult, along with the egg-to-adult survival of the resulting offspring they produce. This is a more comprehensive fitness measure than previous studies estimating selection on SMR. The mating environment also features added structural complexity (see Methods), potentially allowing a greater range of sexual behaviors to be expressed compared to standard *Drosophila* populations that are generally maintained at high density in structurally simple environment (i.e., standard fly vials or bottles). For example, male-mating success may involve searching for females and/or defending a territory, and female can flee when faced with male courtship, all of which are energetically costly and may thus impact SMR. We have previously shown in this population that SMR is both repeatable and differentially correlated with body mass and activity in males versus females (Videlier et al. 2019). Here we used the same high-throughput metabolic system to measure SMR, in addition to body mass and fitness, in close to one thousand separate individuals.

Methods

STOCK POPULATION

A stock population was established in February 2016 from a large sample of a laboratory-adapted population of *D. melanogaster*

that was originally collected in Dundas, ON, in 2006 (MacLellan et al. 2012). Since then, this stock has been maintained with discrete, nonoverlapping generations at 25°C, 50% relative humidity, and with a 12L:12D photoperiod (lights switch at 7 a.m./p.m.) on a standard cornmeal-based food (90 g/l cornmeal, 100 g/l turbinado sugar, 40 g/l yeast, and 12 g/l agar). The population life cycle includes a four-day "mating phase" that takes place in an environment (8 oz culture bottles) with reduced density (10 males and 10 females per bottle) and increased spatial complexity (i.e., dividers inserted into the food and two coiled piper cleaners inside the bottle) compared to standard *Drosophila* maintenance techniques. Males are discarded after the mating phase and females are allowed to lay eggs for 24 hours in standard glass culture vials (28.5 mm × 95 mm). Additional details are provided in Videlier et al. (2019). To create a separate marked "competitor" for use in the fitness assays, in November 2016 a brown eye recessive (*bw*) mutation was introgressed into a copy of the stock population via two rounds of backcrossing. This population was then synchronized with the stock and was maintained in the same way and following the same schedule.

EXPERIMENTAL DESIGN

To quantify selection, both metabolic rate and fitness were measured on individual males and females from the stock population under conditions that closely mimicked their normal maintenance routine. The experiment was performed in six temporal blocks over six generations of the stock population, with each block consisting of three separate temporal subblocks of 32 males and 32 females each (i.e., one subblock per day over three days; see next).

Individuals for use in the assay were raised at four different densities by allowing 2, 5, 10, or 15 stock females to lay eggs in a vial for 24 hours (10 females/vial matches the density during normal maintenance). This was done to increase phenotypic variation in size, and potentially SMR, thereby increasing the power to detect selection. A downside of such a phenotypic manipulation is that it creates the possibility of a density-induced fitness-trait covariance that could be mistakenly interpreted as selection (Rausher 1992; Stinchcombe et al. 2002). In our case this appears unlikely (see Fig. S1 and Discussion). To increase sample size within each block, virgin collection was performed over three consecutive days corresponding to 8, 9, and 10 days after egg laying, creating three groups corresponding to three different "days of emergence." (Nine days after egg laying corresponds to the normal maintenance routine of the stock.) On each day, all newly emerged virgin offspring from the four rearing densities were pooled and then 45 males and 45 females were randomly selected using light CO_2 anesthesia (in the late morning). These flies were subsequently stored, separately by sex, in three vials of 15 within the same incubator as the stock population. At approximately 19:00 hours, 32 females and 32 males were randomly chosen for metabolic rate measurement overnight (remaining individuals were discarded). The following morning, these individuals were weighed (as described next) and then placed in the complex environment for a three-day "mating phase" together with mutant competitor flies (see next), after which females were transferred to new vials for egg laying. Although the stock population normally experiences a four-day mating phase, we used three days so that when the assay females were subsequently transferred to vials for egg laying, they were of the same age as stock females when they lay eggs during regular maintenance.

METABOLIC AND BODY MASS MEASUREMENTS

Metabolic rate measurements were performed following Videlier et al. (2019) using a 64-chamber flow-through respirometry system, housed overnight in a separate incubator. The system consists of four separate units, each comprised of a differential CO_2 analyzer (Li-Cor7000, Li-Cor Biosciences, Lincoln, NE, USA) and a 16-channel flow management, data acquisition, and signal processing system (MAVEn; Sable Systems International, North Las Vegas, NV, USA). Each MAVEn incorporates a flow-distribution manifold, a main board (flow measurement, regulation, and control plus data acquisition and signal processing), and an activity board (sensors for activity, ambient temperature, humidity, and light intensity). A constant stream of dry, CO_2-free air produced by a purge gas generator (PG14L Peak Scientific, Glasgow, Scotland, UK) was split into four different streams, which were pushed through the reference cell of each CO_2 analyzer (Cell A). The air stream was then humidified by flowing through Nafion tubing (du Pont de Nemours and Company, Wilmington, DE, USA) submerged in distilled water, and finally was directed into the flow-distribution manifold where it was physically split into 17 streams (one for each of the 16 chambers and one for the baseline), of which only the baseline was actively regulated at a flow rate of 20 $ml \cdot min^{-1}$. The approximately equivalent flow rates in the nonbaseline channels (range: 15–25 $ml \cdot min^{-1}$) were maintained by means of matched flow resistances based on micro-orifice flow restrictors. A second mass flow meter on the MAVEn's main board measured the actual flow rate of each selected air stream before it was automatically directed through the measurement cell (Cell B) of the CO_2 analyzer.

Before measurement, individuals were chosen randomly from the three sex-specific holding vials and were gently placed, without anesthesia, separately into chambers made of clear plastic tubes (40 mm high by 6 mm diameter). Females and males were placed in odd- and even-numbered chambers, respectively. Measurements were performed for 12 hours overnight, between 19:00 and 7:00 hours, which correspond to the period of

lowest average locomotor activity in this population (Videlier et al. 2019).

Data transformation and extraction were done using ExpeData (Sable Systems International, North Las Vegas, NV, USA). The raw outputs from the activity detectors (one per chamber) were transformed into an index of locomotor activity by first calculating the cumulative sum of the absolute difference between adjacent samples and then by differentiating the resulting channel versus time (equivalent to calculating the slope of the cumulative activity vs. time). The CO_2 trace (one for all of the 16 chambers in a given unit) was corrected for drift using multiple baseline correction measures and was also corrected for a 15-second lag. CO_2 production (VCO_2) was then calculated by multiplying flow rate by the fractional concentration of CO_2. Considering our sampling scheme (~12 hours respirometry run with a 34-minute sampling cycle), each fly was sampled for 120 seconds per sample over a total of 21 separate measurement periods. The first 40 seconds of each measurement was ignored to allow the system to fully equilibrate after changing between chambers. From the remaining 80 seconds we extracted the lowest 20 seconds continuous bouts of VCO_2 using the "nadir" function in ExpeData. In addition to the average of the lowest 20 seconds continuous bout of VCO_2, we also extracted the average flow rate, water vapor, temperature, light intensity, and locomotor activity. We also extracted the average locomotor activity over the 20 seconds immediately prior to the VCO_2 measurement. For each respirometry run, the lowest of the 21 extracted VCO_2 values was selected per individual as their SMR.

The following morning, immediately after each metabolic measurement, body mass was measured by anesthetizing individuals with CO_2 and then weighing them to the nearest 0.001 mg with an MX5Microbalance (Mettler Toledo, Columbus, OH, USA) as described in Videlier et al. (2019). After body mass measurements, individuals were transferred into the fitness assay.

FITNESS ASSAY

Fitness was measured in a competitive assay in which a single focal individual (male or female), which previously had its metabolic rate and body mass measured, was placed together with 9 same-sex *bw* mutant individuals and 10 opposite-sex *bw* individuals in the same "complex" bottle as used during the stock mating phase. Individuals were allowed to interact and mate for three days, after which males were discarded. In the female fitness assay, the single focal female was then transferred to a new vial with fresh media to lay eggs for 24 hours, whereas in the male fitness assay we randomly selected eight of the surviving *bw* females and placed them in pairs in four separate vials with fresh media for egg laying for 24 hours. Brown eye mutant individuals for use in these assays were collected at the same time as the focal individuals and prior to use were housed separately by sex in bottles of 50 individuals within the same incubator.

Female fitness was quantified as the total number of offspring emerging from a vial across two counts performed eight and 10 days after egg laying. (Counting twice reduces the chance of missing individuals that die and are lost in the food.) Focal females that died during the mating phase were assigned a fitness of zero. Male fitness was quantified in the same way except offspring were phenotyped for eye color and counted separately (wild-type red eyes indicating they were sired by the focal male, brown eyes indicating they were sired by a *bw* competitor male). Male fitness was the total number of wild-type offspring produced, although results were qualitatively the same if male fitness was calculated as the proportion of offspring sired by the focal male (unpublished results). Given this, we present only results based on the absolute number of wild-type offspring to avoid additional statistical complexity when dealing with proportions. Although our measure of fitness will be influenced by variation in egg to adult survival of offspring, such mortality was likely low as larvae were raised at low density, so most of the variance in fitness likely originates from differences in survival and fecundity (females) or reproductive success (males; Bateman 1948) of the focal adults themselves.

We attempted to measure all three traits (SMR, mass, and fitness) on 1088 individuals in 17 blocks (64 individuals per block). However, handling errors, equipment problems, and unexplained deaths reduced sample sizes slightly). Individuals with missing values for two of the traits were excluded as they were not informative for estimating covariances (see next). This resulted in a total sample size of 1037 individuals (515 males and 522 females). Of these, 78 individuals had a missing value for one of the traits but were retained because they are informative for estimating the covariance between the other two traits. Repeating the analyses next after excluding these 78 individuals did not qualitatively alter our conclusions.

STATISTICAL ANALYSES

We estimated selection separately in males and females because body mass is sexually dimorphic and previous work on these populations demonstrated that males and females differ in how SMR scales with body size and how activity and SMR covary (Videlier et al. 2019). We applied a modified Lande and Arnold (1983) framework using multivariate models in ASReml-R (Butler et al. 2018) that allowed us to estimate the covariance between fitness, body mass, and SMR while correcting SMR for nuisance parameters that only apply to it (see Supporting Information Methods for R code). The model included relative fitness (absolute fitness divided by its mean) and standardized (mean = 0, SD = 1) body mass and SMR as response variables, and an unstructured (co)variance matrix at the residual level. The

inclusion of one or more fixed effects on a trait will change its residual variance such that it is no longer one, meaning gradients calculated from this will not be standardized gradients. To address this, SMR and body mass were standardized such that their variances (and hence SD) were one after accounting for relevant fixed effect(s) on each. This was done by dividing each trait not by its variance, but by its residual variance obtained from a first fitting a model using the unstandardized traits and the same fixed effects. To control for block and day effects, we fitted a variable that consisted of a unique combination of block (six levels) and day of emergence (three levels) as a fixed effect fitted to all three variables. Fixed effects of temperature, flow rate, and locomotor activity (both 20 seconds before and during SMR measurement) were fitted to SMR only. Light intensity and water vapor were not included because preliminary analyses reveal their effect sizes to be very small. For male fitness, the number of *bw* females that were used for 24 hours of egg laying was also fitted as a continuous effect.

Standardized linear selection differentials (S) were estimated as the covariance between the traits (SMR and mass) and relative fitness from the unstructured residual variance-covariance matrix in the above model. The vector of standardized linear selection gradients (β) on the traits was then estimated as

$$\beta = \mathrm{P}^{-1} S, \quad (1)$$

where $\boldsymbol{S}$ is a vector of selection differentials (on mass and SMR) and $\mathbf{P}$ is the 2×2 phenotypic (co)variance matrix of body mass and SMR (Lande and Arnold 1983). The (co)variances in $\mathbf{P}$ were taken from the larger 3×3 residual covariance matrix from the multivariate model.

To estimate the nonlinear selection gradients, three new second-order "traits" were constructed representing the quadratic (mass^2 and SMR^2) and cross-product terms involving mass and SMR (i.e., mass $\times$ SMR). These terms were then included, alongside relative fitness, SMR, and body mass, in a second multivariate model, yielding a 6×6 phenotypic covariance matrix at the residual level. The same fixed effects applied to SMR were also applied to the second-order terms associated with SMR together with all unique pairwise interactions of these fixed effects. Standardized nonlinear selection gradients (i.e., γs) were estimated as

$$\gamma = \mathbf{P}_2^{-1} \operatorname{cov}(w, \text{traits}), \quad (2)$$

where cov(w, traits) is the vector of covariance between relative fitness and the "traits" (i.e., SMR, mass, SMR^2, mass^2, and SMR$\times$mass) from the unstructured residual variance-covariance matrix and $\mathbf{P_2}$ is the 5×5 phenotypic covariance matrix between SMR, mass, SMR^2, mass^2, and SMR$\times$mass . As for equation (1), $\mathbf{P_2}$ was extracted from the full residual covariance matrix from the multivariate model. Like equation (1), equation (2) is a specific case of the general formula for the least-squares estimates of the partial regression coefficients via matrix algebra (Kendall and Stuart 1973; Morrissey 2014). The partial regression coefficients for the second-order terms were retained as estimates of nonlinear selection, while those for mass and SMR (representing linear selection) were discarded as these are taken from the first-order model (i.e., Eq. 1; Lande and Arnold 1983). Quadratic (but not correlational) gradients were doubled (Stinchcombe et al. 2008).

The overall significance of linear and nonlinear selection were separately tested using a model comparison approach (Chenoweth et al. 2013). For linear selection, a likelihood ratio test (LRT) was used to compare the fit of a "full" multivariate model that included relative fitness, body mass, and SMR and that specified an unconstrained residual covariance matrix, with a "reduced" version of the same model in which the covariances between relative fitness and both SMR and body mass were set to zero. For nonlinear selection, the full model included the three second-order terms (i.e., SMR^2, mass^2, and SMR $\times$ mass) and the reduced model constrained the residual covariances between fitness and the three second-order terms to be zero. To test the significance of the individual selection differentials and gradients (i.e., βs and γs), the appropriate multivariate model was bootstrapped 10,000 times to estimate empirical 95% confidence intervals as the 0.025 and 0.975 quantiles of the distribution of the bootstrapped estimates.

Finally, we analyzed selection separately in males and females for the reasons outlined above but, for completeness, we also compared selection between the sexes. Differences in linear and nonlinear selection between males and females were separately tested using an analogous model comparison approach to that above on a pooled dataset that combined the sexes, treating SMR, mass and fitness in each sex as separate traits. Sex was also included as a fixed effect. The fit of a model with an unconstrained residual covariance matrix was compared with one that specified a "reduced" version in which the covariances between relative fitness and traits (both SMR and body mass for linear selection, and SMR^2, mass^2, and SMR $\times$ mass for nonlinear selection) were constrained to be the same in males and females. In both models nonestimable covariances (i.e., between traits in opposite sexes) were fixed to zero.

Results

In males, there was evidence of linear selection on SMR and body mass overall (LRT: $\chi^2_{2\,\mathrm{df}} = 17.37$, $P < 0.001$; Fig. 1A). Selection differentials on both traits were positive and significant (Table 1). Selection gradients were of somewhat smaller magnitudes than the differentials and had larger 95% CIs and hence

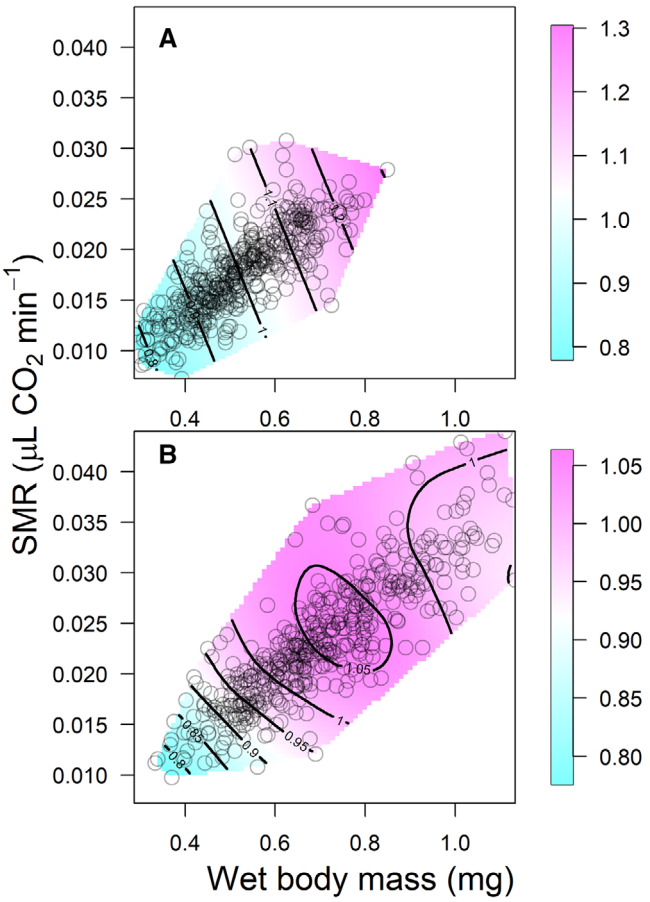

Figure 1. Standard metabolic rate (SMR) as function of wet body mass in (A) 515 male and (B) 522 female *D. melanogaster*. The contour map (thin-plate spline) shows how predicted relative fitness varies as function of SMR and body mass. Points represent individuals.

were not significant (Table 1). Such a pattern is potentially due to collinearity between body mass and SMR ($r = 0.70$; Fig. 1). Finally, there was no evidence of nonlinear selection overall in males (LRT: $\chi^2_{3\,df} = 0.77$, $P = 0.856$; Table 2).

In contrast to males, in females there was no evidence of linear selection overall (LRT: $\chi^2_{2\,df} = 5.21$, $P = 0.074$). Linear selection differentials were smaller than in males and, although individually significant for body mass, both selection gradients were weak and nonsignificant (Table 1). There was, however, statistical support for nonlinear selection overall in females (LRT: $\chi^2_{3\,df} = 8.54$, $P = 0.036$; Fig. 1B), with two of the three nonlinear selection differentials being significant and the third approaching so (Table 2). The estimated gradients suggest that this nonlinear selection arose primarily from stabilizing selection on body mass, but the bootstrapped CI's span zero for the individual gradients, again suggesting collinearity.

Finally, when pooling males and females, the observed difference between the sexes in overall linear (LRT: $\chi^2_{2\,df} = 1.62$, $P = 0.445$) and nonlinear selection (LRT: $\chi^2_{3\,df} = 1.05$, $P = 0.790$) were both nonsignificant. Consistent with this, the 95% CIs of all linear and nonlinear selection gradients overlap between the sexes (Tables 1 and 2).

Discussion

Estimating selection on physiological traits such as SMR is challenging, most notably in small insects, as it involves precisely measuring metabolic rate and fitness in a large number of individuals. Metabolic rate varies substantially within individuals (Nespolo and Franco 2007; White et al. 2013; Auer et al. 2016), necessitating careful attention to controlling for covariates in the design and analysis. Traditionally, selection on metabolic rate has

Table 1. Variance-covariance matrix between relative fitness (*w*), standardized standard metabolic rate (SMR), and standardized body mass in (A) 515 male and (C) 522 female *Drosophila melanogaster* extracted from a three-trait multivariate model.

	(Co)variance matrix			Selection differentials			Selection gradients		
	w	SMR	Mass	*S*	Lower CI	Upper CI	β	Lower CI	Upper CI
(A) Males									
w	0.765	0.144	0.154						
SMR	0.144	1.000	0.701	**0.144**	**0.071**	**0.214**	0.071	−0.040	0.179
Mass	0.154	0.701	1.000	**0.154**	**0.077**	**0.225**	0.104	−0.008	0.213
(B) Females									
w	0.294	0.048	0.056						
SMR	0.048	1.000	0.830	0.048	−0.002	0.094	0.005	−0.084	0.093
Mass	0.056	0.830	1.000	**0.056**	**0.007**	**0.103**	0.052	−0.039	0.142

***Note*: Selection differentials (*S*) were estimated as the covariance between *w* and the trait of interest (values in red), whereas standardized selection gradients (β) were estimated as $\beta = \mathbf{P}^{-1}S$ (eq. 1), where *S* is the vector of selection differentials s) and P is the trait-based phenotypic covariance matrix (blue values). 95% confidence intervals (CIs) are based on 10,000 bootstrap estimates. Bold denotes significant values.**

Table 2. Variance-covariance matrix between relative fitness (*w*), standard metabolic rate (SMR), standardized body mass, and the three variables from second order of SMR and body mass in (A) 515 male and (B) 522 female *Drosophila melanogaster* extracted from a six-trait multivariate model.

	(Co)variance matrix						Selection differentials			Selection gradients		
	w	SMR	Mass	SMR^2	$Mass^2$	SMR × Mass	*C*	Lower CI	Upper CI	γ	Lower CI	Upper CI
(A) Males												
w	0.765	0.145	0.154	−0.044	0.023	−0.013						
SMR	0.145	1.001	0.701	0.216	0.230	0.210						
Mass	0.154	0.701	1.000	0.197	0.481	0.304						
SMR^2	−0.044	0.216	0.197	3.909	1.982	2.760	−0.044	−0.196	0.102	−0.036	−0.246	0.152
$Mass^2$	0.023	0.230	0.481	1.982	3.725	2.712	0.023	−0.126	0.171	0.001	−0.154	0.166
SMR × Mass	−0.013	0.210	0.304	2.760	2.712	2.950	−0.013	−0.132	0.109	−0.005	−0.147	0.145
(B) Females												
w	0.294	0.048	0.056	−0.077	−0.133	−0.101						
SMR	0.048	1.000	0.830	0.565	0.409	0.469						
Mass	0.056	0.830	1.000	0.454	0.486	0.444						
SMR^2	−0.077	0.565	0.454	3.824	2.516	3.099	−0.077	−0.185	0.022	−0.019	−0.266	0.227
$Mass^2$	−0.133	0.409	0.486	2.516	3.642	2.993	**−0.133**	**−0.233**	**−0.038**	−0.121	−0.321	0.108
SMR × Mass	−0.101	0.469	0.444	3.099	2.993	3.071	**−0.101**	**−0.194**	**−0.013**	0.024	−0.205	0.233

***Note*: Nonlinear standardized selection gradients were estimated as $\gamma = P_2^{-1}\text{cov}(w, \text{traits})$ (eq. 2), where cov is the vector of covariance between relative fitness (*w*) and traits (red values) and P_2 is the trait-based 5 × 5 phenotypic covariance matrix (blue values). 95% confidence intervals (CIs) are based on 10,000 bootstrap estimates. Bold denotes significant values.**

been estimated while "correcting" for body mass, either by using mass-specific values (i.e., per unit mass) or by taking the residuals from a regression of metabolic rate on mass. However, such approaches are unable to separate the traits under selection (i.e., body mass, SMR, or both; Hayes 2001; Hagmayer et al. 2020), they ignore the possibility of correlational selection, and they can involve doing "statistics on statistics" that can fail to propagate uncertainty and may result in statistical bias (Garcia-Berthou 2001; Morrissey 2014). Measuring fitness can also be challenging and past studies have tended to rely on components thereof. Although useful for understanding how selection arises, this can provide biased insight into net selection.

Here, we performed high-throughput respirometry on individuals from a laboratory population of *D. melanogaster* with a life cycle that facilitated comprehensive measures of fitness in both sexes. Our fitness measure integrated adult survival, reproductive success, and fecundity, as well as the viability to adult emergence of resulting offspring, all in an abiotic and social environment that was extremely similar to that which the population was adapted. Using these data, we employed a multivariate modeling approach to estimating linear and nonlinear selection while controlling statistically for nuisance variables specific to each trait. Our results provide evidence of linear selection on body mass and/or SMR in males, and nonlinear selection primarily on body mass in females. Despite substantial sample sizes (515 males and 522 females), the partitioning of selection between these two highly correlated traits remained challenging.

In males, linear differentials on body mass and SMR were both positive and significant, indicating direct and/or indirect selection for increased values of these traits. Selection gradients, which quantify selection on each trait while controlling for the other traits in the model, were of somewhat smaller magnitudes to the differentials and were slightly stronger for mass compared to SMR (Table 1). Although the individual gradients were not significant based on approximate 95% CIs, they approached so, in particular for mass (i.e., the lower bound of the 95% CI just crossed zero). Notably, the 95% CIs for the gradients are 50% wider than those for the differentials, reflecting increased uncertainty in partitioning selection in the face of a strong correlation between these traits (Fig. 1).

With the above caveat in mind, the point estimates of our gradients suggest moderately strong directional selection on body mass and SMR in males (median standardized phenotypic gradients from a review of selection in nature is |0.18|; Kingsolver et al. 2001), and little evidence of nonlinear selection including correlational (i.e., SMR × body mass gradient; Table 2). It is therefore worth considering why selection may favor increased values of each these traits independent of the other. For body mass, sexual selection is one possibility if increased mass leads to greater reproductive success. Increased mating success of larger males

has sometimes, but not always, been observed in *Drosophila* (e.g., Partridge and Farquhar 1983; Partridge et al. 1987; Santos et al. 1988; Pitnick 1991; Baxter et al. 2018, but see Markow et al. 1996; Bangham et al. 2002). Larger males may also have higher postcopulatory success (Pitnick and Markow 1994; Bangham et al. 2002). Compared to standard *Drosophila* lab stocks, our population was also adapted to a lower density mating environment with added structural complexity. This may provide increased opportunity for males to defend food/egg laying substrates as a way to access females, and larger males tend to have an advantage in such territorial interactions in *Drosophila* (Hoffmann 1987; White and Rundle 2014).

With respect to SMR, increased values correspond to males with higher metabolic maintenance costs, which can be seen as the "idling" cost of an individual's metabolic machinery. As such, males with higher SMR may have more energy available to allocate to costly behaviors or physiological processes. Why might selection favor this? Again, it is possible that such males have increased mating success if they are better at defending a territory and/or searching for, pursuing and courting females. These demands may be enhanced in our lower density, structurally complex mating environment in which females can hide and escape male courtship. Indeed, similar manipulations of the mating environment in *D. melanogaster* have been shown to reduce the frequency of sexual interactions and mating, and to increases female feeding rates (Yun et al. 2017; Fig. S1 in Yun et al. 2019). Previous work with the current population also revealed a positive correlation between resting metabolic rate and locomotor activity in males (Videlier et al. 2019), suggesting that individuals that perform more energetically demanding activities tend to have elevated maintenance costs.

In females, nonlinear selection was significant overall, indicating curvature of the fitness surface. This appeared to arise in large part from stabilizing selection on body mass although the individual quadratic and correlational gradients were nonsignificant (Table 2), probably because collinearity will be even more problematic for second-order traits. Nevertheless, the point estimates for body mass was negative and substantially larger than that for SMR or the correlational gradient (Table 2). The nonparametric fitness surface supports this and reveals a fitness peak within the upper range of mass values (Fig. 1B).

Although the fitness surface and selection differentials suggest directional selection for both body mass and SMR over much of the phenotypic range in females (i.e., for trait values below the peak), our estimated gradients indicate that this selection on SMR is largely indirect, arising from its correlation with body mass (i.e., gradients on SMR are weak in Tables 1 and 2). Why might selection favor increased female body size? Fecundity selection seems likely as there is a strong positive association between body size and egg production in *Drosophila* (Lefranc and Bundgaard 2000; Byrne and Rice 2006). It is less obvious as to why fitness may decline at high body mass, although this could represent a trade-off in energy allocation if the energetic costs of further increases in mass come at the expense of greater investment in fecundity. A recent result in this population suggests the presence of allocation trade-offs in females, as reflected by a negative correlation between resting metabolic rate and locomotor activity at the beginning of the night (Videlier et al. 2019), a time which may correspond to a peak in egg laying (Manjunatha et al. 2008).

At first glance, the contrasting significance of linear versus nonlinear selection in males versus females suggests sex-specific selection on these traits. However, these differences were not significant, likely reflecting in part the similarity of the fitness surfaces for overlapping trait values between the sexes (Fig. 1; the curvature in females occurs at trait values greater than those observed in males). It is therefore possible that males of a similarly large size would likewise experience reduced fitness, but in the absence of such phenotypes we do not know. Further phenotypic manipulation to generate an even broader range of male phenotypes would be necessary to resolve this. Phenotype manipulations can also be useful in reducing or eliminating collinearity among traits (Sinervo 1990; Campbel 2009), allowing combinations of traits to be created that would otherwise be rare or nonexistent. In this case, however, it is unclear how mass could be manipulated independently of SMR. A potential downside of a phenotypic manipulation like density is that it can affect all traits, including fitness, and it therefore creates the possibility of an environmentally (i.e., density) induced fitness-trait covariance that can be mistaken for selection (Rausher 1992; Stinchcombe et al. 2002). Increased density slows development and thus delays adult emergence in *Drosophila*. Day of emergence was included as a fixed effect in all our analyses, so to the extent that density and emergence day covary, our analysis accounts for density effects. In addition, neither male nor female fitness varied significantly by day of emergence (Fig. S1), strongly suggesting that the selection we observed was not the result of a density-induced fitness-trait covariance.

Lande and Arnold (1983) provide a framework for quantifying selection via multivariate regression but problems arise when unique covariates apply to different traits, including fitness. Here we outlined an approach that allows trait-specific covariates by extracting phenotypic covariance matrices at the residual level from a multivariate model of traits and fitness. Linear selection differentials are given by the covariance between fitness and each standardized trait, and linear selection gradients are estimated as the product of the linear selection differentials and the inverse of a subset of the full phenotypic covariance matrix (**P**) that excludes fitness as a trait (Lande and Arnold 1983). The latter is simply the least-squares estimates of the partial regression

coefficients obtained via matrix algebra (Kendall and Stuart 1973), meaning this approach can be extended to estimating nonlinear gradients simply by including the squared traits and their second-order interactions in the multivariate model. This is preferable to Equation (14a) in Lande and Arnold (1983), which provides an approximation of the nonlinear gradients under certain assumptions. To our knowledge, this statistical approach to estimating nonlinear selection has not been previously employed.

White et al. (2019) recently put forward correlational selection as an explanation for the widely observed metabolic scaling allometry. For correlational selection to occur, particular combinations of SMR and mass must be advantageous over other combinations and, over time, correlational selection change trait covariance (Sinervo and Svensson 2002). In particular, correlational selection favoring small and large individuals with, respectively, high and low mass-specific SMR would the give rise to the widely observed sublinear scaling of SMR with mass. Using a simulation approach combined with interspecific data, White et al. (2019) concluded that the scaling allometry between metabolic rate and body mass arose as a consequence of correlational selection on these traits. In our study, however, we did not detect correlational selection on SMR and body mass, but more research is needed to estimate the possibility of nonlinear trait-fitness covariance at the genetic level.

Finally, as with any observational selection analyses, confounding effects of environmentally induced covariances between traits and fitness can be mistaken for selection (Rausher 1992; Stinchcombe et al. 2002). This includes potential effects of density discussed above, but also other unidentified environment variables that could affect traits and fitness. The problem of environmentally-induced covariances can be overcome via a breeding design that estimates selection at the genetic level. Estimating the quantitative genetic architecture of fitness and SMR may also provide a direct test of the possibility of sexual conflict over metabolic rate.

AUTHOR CONTRIBUTIONS

VC and HDR conceived the project; MV, VC. and HDR contributed to study design; and MV collected the data. MV and VC analyzed the data with input from AJW and HDR, and all authors wrote the manuscript.

ACKNOWLEDGMENTS

B. Martire helped in the laboratory. We thank S.J. Arnold and J.G.A. Martin for helpful discussions about quantifying selection. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (HDR and VC) and from the Canada Foundation for Innovation (VC).

DATA ARCHIVING

Data can be found on Dryad (https://doi.org/10.5061/dryad.63xsj3v17); R-script is provided in the Supporting Information.

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Associate Editor: J. W. McGlothlin
Handling Editor: T. Chapman

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Standard metabolic rate (SMR) as function of wet body mass in A) 515 male and B) 522 female *D. melanogaster* that emerged on day 8 (red squares), day 9 (green dots), or day 10 (blue triangles) after egg laying.