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# Genetic modulation of energy metabolism in birds through mitochondrial function

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Despite their central importance for the evolution of physiological variation, the genetic mechanisms that determine energy expenditure in animals have largely remained unstudied. We used quantitative genetics to confirm that both mass-specific and whole-organism basal metabolic rate (BMR) were heritable in a captive-bred population of stonechats (*Saxicola torquata* spp.) founded on birds from three wild populations (Europe, Africa and Asia) that differed in BMR. This argues that BMR is at least partially under genetic control by multiple unknown nuclear loci each with a limited effect on the phenotype. We then tested for a genetic effect on BMR based on mitochondrial–nuclear coadaptation using hybrids between ancestral populations with high and low BMR (Europe–Africa and Asia–Europe), with different parental configurations ($female_{high}$–$male_{low}$ or $female_{low}$–$male_{high}$) within each combination of populations. Hybrids with different parental configurations have on average identical mixtures of nuclear DNA, but differ in mitochondrial DNA because it is inherited only from the mother. Mass-specific BMR differed between hybrids with different parental configurations, implying that the combination of mitochondrial and nuclear DNA affected metabolic rate. Therefore, our findings implicate mitochondrial function as an important regulator of energy metabolism. In combination with the substantial heritabilities of metabolic rate, and corroborated by genetic differences in the mitochondrial genome, these results set the stage for further investigations of a genetic control mechanism involving both mitochondrial and nuclear genes determining metabolic rate at the whole-organism level.

**Keywords:** metabolic rate; heritability; selection; mitochondrial DNA; intergenomic coadaptation; mitochondrial respiration

## 1. INTRODUCTION

The genetic mechanisms that determine energy expenditure in animals are largely unknown, despite the central importance of metabolic rate for behaviour, ecology and life history of organisms. Some studies report a heritable component (Lacy & Lynch 1979; Sadowska *et al.* 2005; Rønning *et al.* 2007) to basal metabolic rate (BMR), the minimum rate of metabolism measured at rest, in the rest phase and in the thermal neutral zone (King 1974), whereas others do not (Dohm *et al.* 2001; Nespolo *et al.* 2003, 2005), and few demonstrate a direct response to selection on BMR (Ksiazek *et al.* 2004). These results imply that at least some within-species variation in BMR has a genetic basis, although the genetic mechanisms remain unclear. Recent analyses of heritabilities and genetic correlations among body mass, whole-organism BMR and mass-specific BMR in three populations of stonechats (*Saxicola torquata* spp.) have showed that these traits can evolve independently (B. I. Tieleman, M. A. Versteegh, B. Helm & N. J. Dingemanse 2006, unpublished data). These results raise the possibility that metabolic rate is influenced by multiple genes.

Since BMR, as measured by oxygen consumption, is a product of summed mitochondrial oxygen consumption, mitochondrial function provides an important link between cellular processes and whole-organism metabolic rate, and, in addition, implicates a limited set of candidate genes to impart genetic control. After accounting for body size, variation in BMR has been attributed to differences in size of metabolically highly active organs (Daan *et al.* 1990; Konarzewski & Diamond 1995), mitochondrial density (Else & Hulbert 1985), inner mitochondrial membrane surface area and/or degree of unsaturation of lipids in membranes affecting proton permeability (Hulbert & Else 2005). However, studies that failed to confirm that organ size accounted for variation in BMR (Song & Wang 2002; Tieleman *et al.* 2003; Russell & Chappell 2007), or that variation in lipids in membranes was associated with BMR (Brookes *et al.* 1997; Brzek *et al.* 2007), leave us with a poor understanding of the connections between cellular processes and whole-organism energy expenditure.

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Mitochondria consume approximately 90 per cent of respired oxygen during the production of ATP, the molecule that powers such processes as protein synthesis, ion gradients and actinomyosin ATPase (Rolfe & Brown 1997; Nichols & Ferguson 2002). The myriad of chemical reactions that supply electrons to the electron transport system in mitochondria produce heat as a by-product that contributes to thermoregulation in endotherms (Rolfe & Brown 1997; Nichols & Ferguson 2002). Within mitochondria, the electron transport system consists of large protein complexes (complexes I–V) that sequentially transport electrons and create a proton-motive force that drives ATP synthesis. The electron transport system contains 13 subunits coded by the mitochondrial genome, in addition to at least 70 nuclear-encoded peptides that are imported into the mitochondria and assembled together with the mitochondrially encoded subunits to produce functional complexes (Blier *et al.* 2001; Ballard & Rand 2005; Das 2006). Proteins containing mitochondrial DNA-encoded polypeptides play crucial roles in the regulation of mitochondrial respiration, in complexes I, III and IV, and in the production of ATP in complex V (Davey & Clark 1996; Bai *et al.* 2000).

Maternally inherited, mitochondrial DNA has traditionally been used as neutral marker in phylogeographic studies (Zink & Barrowclough 2008), but an increasing number of studies support the idea that climatic conditions can provide a selective ecological gradient that alters mitochondria haplotype, potentially changing mitochondria function and heat production (Matsuura *et al.* 1993; Mishmar *et al.* 2003; Ballard & Whitlock 2004; Ruiz-Pesini *et al.* 2004; Ballard & Rand 2005; Zink 2005; but see Elson *et al.* 2004). Furthermore, Fontanillas *et al.* (2005) showed a relationship between mitochondrial haplotype and maximal non-shivering thermogenesis in white-toothed shrews from a lowland and an upland population, suggesting selection on mitochondria in these two populations.

We exploited differences in inheritance of mitochondrial and nuclear genes to obtain insights into the mechanisms of genetic influence on metabolic rate. Using a captive-bred population of stonechats (*S. torquata* spp.) founded on birds from three distinct wild populations with different BMRs (from, in decreasing BMR order, Asia, Europe and Africa; Klaassen 1995; Wikelski *et al.* 2003; Tieleman 2007), we first applied quantitative genetics analyses to evaluate the heritable basis of BMR in our dataset of combined inter- and intrapopulation variations. The heritabilities and genetic correlations are compared with previous analyses for each of the three ancestral populations separately (B. I. Tieleman, M. A. Versteegh, B. Helm & N. J. Dingemanse 2006, unpublished data). We then tested three hypotheses about mechanisms of genetic influence on BMR using two types of hybrids, European–African and Asian–European crosses, with reciprocal parental configurations, $\text{Europe}_{\text{female}}$–$\text{Africa}_{\text{male}}$ versus $\text{Africa}_{\text{female}}$–$\text{Europe}_{\text{male}}$ and $\text{Asia}_{\text{female}}$–$\text{Europe}_{\text{male}}$ versus $\text{Europe}_{\text{female}}$–$\text{Asia}_{\text{male}}$. *A priori*, we envisioned three possible outcomes: (i) No difference in metabolic rate between hybrids with contrasting parental configurations and hybrid metabolic rates intermediate between ancestral values. This would indicate that metabolic rate results predominantly from nuclear DNA-encoded processes ('nuclear control' hypothesis). (ii) Metabolic rate differs between hybrids with contrasting parental configurations and resembles the mother's metabolic rate, pointing to dominance of the mitochondrial DNA-encoded components of the electron transport system in determining metabolism ('mitochondrial control' hypothesis). (iii) Metabolic rate differs between hybrids with contrasting parental configurations and does not resemble the mother's metabolic rate, providing evidence for the importance of a match between mitochondrial and nuclear genomes to regulate metabolic rate ('mitochondrial–nuclear coadaptation' hypothesis; Rand *et al.* 2004). Next, we added quantitative genetics analyses to explore maternal and paternal effects on metabolic rate. Finally, we compared sequences of all mitochondrial protein-coding genes between the European and African populations to confirm the presence of amino acid-changing genetic differences in the mitochondrial genome.

## 2. MATERIAL AND METHODS

### (a) *Birds and housing*

Stonechats are small passerines with a wide geographical distribution in Europe, Africa and Asia, ranging from 71° N to 35° S (Urquhart 2002). We studied stonechats from three different populations belonging to three different subspecies, from Europe (Austria, *Saxicola torquata rubicola*), Africa (Kenya, *Saxicola torquata axillaris*) and Asia (Kazakhstan, *Saxicola torquata maura*), in addition to European–African hybrids and European–Asian hybrids. The phylogenetic relationships among these populations and subspecies have been described in detail in Illera *et al.* (2008). Although our birds came from geographically separate locations, they produce fertile hybrids when interbreeding in captivity. Our study design provided us with two independent tests of the effect of the parental configuration within a lineage on mass-specific BMR of hybrids. For the European–African combination, we measured 80 individuals of four different genotypic pairings, comprising 45 pure Europeans, 14 pure Africans, 11 hybrids with European mothers and 10 hybrids with African mothers. For the study of Europeans and Asians, we measured 29 individuals in addition to the 45 pure Europeans: 15 pure Asians, 5 hybrids with European mothers and 9 hybrids with Asian mothers. Because birds that we measured varied in age from 8 months to 8 years, we incorporated age as a factor in the analyses. Individuals were kept in separate cages, in rooms with controlled temperature (20–22°C) and day length as experienced by European birds in the winter quarters (40° N). We measured each individual one to three times during winter (total 131 measurements), in the periods 9–13 February 2005 or 24 November 2005–13 February 2006. During these periods none of the birds were breeding, moulting or showing migratory restlessness, the latter verified by recordings of night-time activity (Helm *et al.* 2005).

### (b) *Metabolic rates*

#### (i) *Laboratory set-up and metabolic measurements*

One hour before the start of experiments, we removed water and food from cages to ensure birds were post-absorptive (B. I. Tieleman 2005, personal observation). We placed birds in 13.5 l metal metabolic chambers with Plexiglas lids that closed airtight. In the metabolic chambers, birds perched on a wire mesh platform placed over a layer of paraffin oil. We set

the metabolic chambers inside a climatic chamber with a constant temperature of 35°C ± 0.5°C, a temperature within the thermoneutral zone of European stonechats (Tieleman 2007).

We used standard flow through respirometry to measure $O_2$ consumption of stonechats (Gessaman 1987). Compressed air was pumped through columns of silica gel–soda lime–silica gel to remove water and $CO_2$, through calibrated (Levy 1964) mass-flow controllers (model 5850E, Brooks, Hatfield, PA, USA) set at 500 ml $min^{-1}$, and then into the chambers.

Air that exited the chamber passed through columns of silica gel–ascarite–silica gel, before entering an oxygen analyser (Oxzilla, Sable Systems, Las Vegas, NV, USA) to measure the fractional concentration of oxygen of the air stream. A stream of dry $CO_2$-free air was used as reference air. We recorded atmospheric pressure, air temperature inside each metabolic chamber and delta $O_2$ with a data logger (model CR23X, Campbell Scientific, Logan, UT, USA).

At least 3 hours after birds were placed in the metabolic chambers, and when $O_2$ concentration had been stable for at least 10 min, we calculated the lowest running average over 10 min of data for BMR (Tieleman 2007). To calculate BMR from $O_2$ consumption, we used eqn (2) from Hill (1972) using a conversion factor of 20.1 J $ml^{-1}$ $O_2$ (Schmidt-Nielsen 1997). Birds were weighed immediately before and after metabolic measurements, and average weights are used in analyses.

(ii) *Statistical analysis of metabolic rates*

We analysed data for BMR from the European–African hybrids and the European–Asian hybrids separately. For each dataset, we divided birds into four groups—pure Europeans, hybrids with European mothers, hybrids with African (or Asian) mothers and pure Africans (or Asians)—and tested differences in BMR among groups. We used a mixed model with individual entered as random effect to incorporate multiple measurements on one-third of the individuals (using MLwiN v. 2.02). Age, sex and body mass were included as fixed effects, and we used backward elimination of non-significant fixed effects ($p > 0.05$) as our selection procedure (Crawley 1993). We tested all two-way interactions but do not report non-significant results. Age had no significant effect in any of the models (all $p > 0.29$); we therefore omitted age from the models.

### (c) *Calculation and interpretation of mass-specific BMR*

We calculated mass-specific BMR as whole-organism BMR divided by body mass. We interpreted this value as the product of the biochemical processes of an average gram of tissue, i.e. average tissue-specific metabolic rate (Speakman 2005; Tieleman *et al.* 2006). We assume that body composition and mitochondrial density are the same in the different groups of stonechat hybrids, and interpret differences in mass-specific BMR to result from differences in mitochondrial function. We find it unlikely that the parental configuration of the hybrids would affect body composition or mitochondrial density, but emphasize that we have no data on body composition or mitochondrial density. Hence, these assumptions remain to be tested.

### (d) *Quantitative genetics analysis with animal models*

(i) *Pedigrees*

Wild-caught stonechats have been added to our captive population over a 15-year period. For our quantitative genetics analyses, we assumed that wild-caught nestlings collected from different nests were not related to each other, and that nestlings collected from the same nest had the same genetic father and mother. In captivity, stonechats bred in aviaries that contained a single pair, thus for all offspring the genetic father and mother were known. After hatching, chicks were fed by the mother for approximately 5 days and thereafter hand-raised. We had a panmictic population of stonechats with known relationships among birds, including crosses among generations and among populations. The resulting pedigree included 50 African, 127 European and 53 Asian individuals, in addition to 21 European–African hybrids and 14 European–Asian hybrids. The birds that we measured belonged to generations 0 (wild-caught) to 4, with the largest numbers from generations 1 and 2. To indicate the genetic basis for variation within the captive populations, we calculated the number of individuals from the wild that produced the first generation of captive individuals: 20 African, 43 European and 18 Asian stonechats (Lynch & Walsh 1998).

(ii) *Estimation of genetic parameters and statistical analysis*

We estimated variance components, heritabilities and genetic correlations with restricted maximum-likelihood 'animal models' (Falconer & Mackay 1996; Kruuk 2004) using the program ASReml v. 2.0 (Gilmour *et al.* 2006). We used these animal models to partition the total phenotypic variance ($V_P$) of a trait into additive genetic variance ($V_A$) and residual variance ($V_R$). The model included sex as a fixed effect, accounting for differences between sexes in phenotypic means, and animal as random effect, taking into account repeated measures. Heritability ($h^2$) was calculated as $V_A/V_P$ (Falconer & Mackay 1996). We used bivariate animal models to estimate additive and residual covariances and correlations ($r$). To test whether $V_A$ was significantly larger than zero, we compared the $-2\times$log likelihood of a model that estimated $V_A$ with one that did not. To test whether the estimates of $r_A$ were significantly different from zero (or from 1 or $-1$), we compared the $-2\times$log likelihood of models with $V_A$ fixed, and $r_A$ either fixed at zero (or 1 or $-1$) or unconstrained. The corresponding significance levels were taken from a $\chi^2$ distribution. To allow comparisons of additive genetic and residual variances across traits, we calculated coefficients of additive genetic variance ($CV_A$) and residual variance ($CV_R$) following Houle (1992).

The maternal inheritance of mitochondria might lead to a maternal genetic effect on offspring metabolism (mitochondrial control hypothesis). To estimate the maternal genetic effects, we constructed an animal model that included mother as additional random effect with the associated variance–covariance matrix determined by the additive genetic relatedness matrix (Kruuk 2004). Likewise, we tested maternal environmental effects (by fitting maternal identity as random effect), paternal genetic and environmental effects, and permanent environment effects (following Kruuk 2004). We calculated the $\chi^2$-distributed difference between the $-2\times$log likelihoods of a model with the effect and one without to establish statistical significance.

Table 1. Quantitative genetics parameters for body mass, whole-organism basal metabolic rate (BMR) and mass-specific BMR in stonechats (combined from Europe, Africa and Asia, including hybrids). (*a*) Estimates of additive genetic variance ($V_A$), residual variance ($V_R$), heritability ($h^2$), coefficient of additive genetic variation ($CV_A$) and coefficient of residual variation ($CV_R$). *p*-values (written as exponents) indicate one-tailed significance of difference from zero for estimates of $V_A$. Sex was included as fixed factor for all traits (see §2). (*b*) Phenotypic correlations (in italics) and additive genetic correlations ($r_A$). Above the diagonal, *p*-values denote two-tailed significance of differences from zero ($p_0$) for estimates of $r_A$, and one-tailed significance from 1 ($p_1$) or −1 ($p_{-1}$).

| trait | phenotypic mean (s.d.) | $V_A$ (s.e.) | $V_R$ (s.e.) | $h^2$ (s.e.) | $CV_A$ | $CV_R$ |
|---|---|---|---|---|---|---|
| (*a*) | | | | | | |
| body mass (g) | 14.9 (2.29) | 3.277 (0.679)$^{<0.0001}$ | 1.389 (0.253) | 0.702 (0.067) | 12.1 | 7.9 |
| BMR (kJ d$^{-1}$) | 22.2 (2.23) | 1.857 (0.818)$^{0.003}$ | 3.345 (0.599) | 0.357 (0.130) | 6.1 | 8.2 |
| mass-specific BMR (kJ d$^{-1}$ g$^{-1}$) | 1.51 (0.215) | 0.035 (0.0077)$^{<0.0001}$ | 0.015 (0.0029) | 0.694 (0.073) | 12.4 | 8.2 |

| | body mass (g) | BMR (kJ d$^{-1}$) | mass-specific BMR (kJ d$^{-1}$ g$^{-1}$) |
|---|---|---|---|
| (*b*) | | | |
| body mass (g) | — | $p_0=0.20$<br>$p_1<0.0001$ | $p_0<0.0001$<br>$p_{-1}=0.001$ |
| BMR (kJ d$^{-1}$) | *0.294* (*0.084*)<br>0.264 (0.195) | — | $p_0=0.12$<br>$p_1<0.0001$ |
| mass-specific BMR (kJ d$^{-1}$ g$^{-1}$) | *−0.703* (*0.048*)<br>−0.826 (0.073) | *0.443* (*0.076*)<br>0.291 (0.205) | — |

### (e) *Mitochondrial DNA sequences*

#### (i) *Generation of mtDNA sequences*

DNA was isolated from muscle tissue from two African and two European individuals using a standard phenol–chloroform procedure. Sequences for all coding genes were obtained by amplifying gene regions in roughly 500 bp sections using primers designed by M. Sorenson for sequencing mtDNA in birds (described at http://people.bu.edu/msoren/primers.html), supplemented with additional primers designed by O. Haddrath and A. J. Baker. Both strands of each amplicon were sequenced using a BigDye (v. 3.0) Cycle Sequencing Kit (Applied Biosystems). Cycle-sequenced products were cleaned using Sephadex columns and nucleotide sequences determined using an ABI 3100 genetic analyser.

#### (ii) *Analyses of mitochondrial DNA*

Because we were interested in differences among populations in mitochondrial proteins as functional components in the electron transport system, we focused on a combined analysis of genes that contribute proteins to four of the five metabolic complexes in the electron transport system (Davey & Clark 1996; Korzeniewski & Mazat 1996; Bai *et al.* 2000): complex I (NADH 1–6); complex III (cytochrome *b*); complex IV (COI–III); and complex V (ATP6 & 8). We explored whether the sequence differences between the European and African populations resulted in (possibly functional) amino acid replacements or in non-functional, synonymous, variation. We present results for individual mitochondrial genes in appendix 1 of the electronic supplementary material.

Finally, we looked for evidence of selection leading to divergence in mitochondrial DNA among stonechat populations, using McDonald–Kreitman (MK) tests (McDonald & Kreitman 1991) to compare the ratio of amino acid replacement variation to synonymous variation within species with the ratio of replacement to synonymous divergence between taxa. Under neutrality these two ratios should be equal, whereas under different types of selection they should differ from one another. The direction and degree of departures from this neutral prediction were quantified with the neutrality index, NI (Rand & Kann 1996). Assuming that synonymous sites are evolving neutrally, NI < 1.0 indicates an excess of amino acid divergence, which is inferred to result from positive selection, whereas NI > 1.0 indicates an excess of amino acid polymorphism resulting in purifying selection.

## 3. RESULTS

### (a) *Heritabilities and genetic correlations of body mass and metabolic rate*

Estimates of $h^2$ for mass-specific BMR, whole-organism BMR and body mass varied between 0.36 and 0.70, and all additive genetic variances were significantly larger than zero (table 1). Furthermore, all genetic correlations were significantly different from 1 (or −1 for mass versus mass-specific BMR), indicating that these variables could evolve at least partly independently of each other (table 1).

### (b) *Hybrids with different parental configurations differ in metabolic rate*

Mass-specific BMR of hybrid stonechats with different parental configurations differed from each other in both the European–African and the Asian–European pairings (table 2; figure 1). Furthermore, our results confirmed differences in mass-specific BMR, whole-organism BMR and body mass among stonechats of different origin (table 2; figure 1). Analysis of the European–African dataset (figure 1*a*) showed that mass-specific BMR was significantly affected by group ($\chi^2_3=35.88$, $p<0.0001$) and by sex ($\chi^2_1=8.76$, $p=0.003$). *Post hoc* analysis supported the mitochondria–nuclear coadaptation hypothesis and failed to support the mitochondrial control and nuclear control hypotheses: the two

Table 2. Body mass, whole-organism and mass-specific basal metabolic rate (BMR) for four groups of stonechats with different parental configurations, originating from (*a*) the African and European populations and (*b*) the European and Asian populations. Values are average ±1 s.d. for all measurements per group. *N*, number of individuals; *n*, number of measurements.

| parental configuration (mother×father) | body mass[a] (g) | whole-organism BMR[b] (kJ d$^{-1}$) | mass-specific BMR (kJ d$^{-1}$ g$^{-1}$) | *n*, *N* |
|---|---|---|---|---|
| (*a*) | | | | |
| African×African | 18.0±2.44a | 22.3±1.83 | 1.26±0.144 | 19, 14 |
| African×European | 15.0±1.39b | 22.3±1.33 | 1.50±0.191 | 18, 10 |
| European×African | 16.1±2.89c | 21.9±2.24 | 1.38±0.166 | 22, 11 |
| European×European | 14.3±1.73b | 22.0±2.34 | 1.55±0.184 | 72, 45 |
| **parental configuration (mother×father)** | **body mass[c] (g)** | **whole-organism BMR[d] (kJ d$^{-1}$)** | **mass-specific BMR (kJ d$^{-1}$ g$^{-1}$)** | ***n*, *N*** |
| (*b*) | | | | |
| European×European | 14.3±1.73a | 22.0±2.34 | 1.55±0.184 | 72, 45 |
| European×Asian | 14.0±1.30ab | 24.6±2.77 | 1.76±0.093 | 5, 5 |
| Asian×European | 14.2±1.30ab | 21.7±1.54 | 1.55±0.201 | 10, 9 |
| Asian×Asian | 13.1±1.39b | 21.8±2.98 | 1.67±0.223 | 18, 15 |

[a] Body mass was significantly affected by group ($\chi^2_3=38.95$, $p<0.0001$) and sex ($\chi^2_1=16.52$, $p<0.0001$). Identical letters following values indicate non-significant differences between groups, with a *post hoc* test.
[b] Whole-organism BMR: without including body mass as covariate, whole-organism BMR was not significantly affected by group ($\chi^2_3=0.34$, $p=0.95$) or sex ($\chi^2_1=2.59$, $p=0.11$). When including body mass as covariate, the model included the interaction sex×body mass ($\chi^2_1=5.90$, $p=0.015$), body mass ($\chi^2_1=11.22$, $p=0.0008$), sex ($\chi^2_1=5.56$, $p=0.018$) and group ($\chi^2_3=9.37$, $p=0.025$).
[c] Body mass was significantly affected by group ($\chi^2_3=13.71$, $p=0.003$) and sex ($\chi^2_1=19.45$, $p<0.0001$). Identical letters following values indicate non-significant differences between groups, with a *post hoc* test.
[d] Whole-organism BMR: without including body mass as covariate, whole-organism BMR was not significantly affected by group ($\chi^2_3=6.92$, $p=0.07$), but significantly differed between sexes ($\chi^2_1=5.73$, $p=0.0017$). When including body mass, the model that remains after eliminating non-significant effects of sex ($\chi^2_1=0.72$, $p=0.40$) and group ($\chi^2_3=7.30$, $p=0.063$) contains only mass ($\chi^2_1=14.46$, $p=0.0001$).

hybrid groups differed significantly from each other and from their maternal pure lines (African×European versus African×African, $\chi^2_1=19.18$, $p<0.0001$; European×European versus European×African, $\chi^2_1=12.27$, $p=0.0005$; European×African versus African×European, $\chi^2_1=5.96$, $p=0.015$).

The analysis of the European and Asian stonechats duplicated the group effects, but not the sex effects, found in the European and African combinations: group significantly affected mass-specific BMR ($\chi^2_3=9.56$, $p=0.023$), but sex did not ($\chi^2_1=2.80$, $p=0.09$; after deleting the interaction group×sex, $\chi^2_3=7.35$, $p=0.062$). *Post hoc* analysis revealed significant differences between both hybrid types (European×Asian versus Asian×European, $\chi^2_1=4.15$, $p=0.04$) and did not support a maternal effect that would indicate mitochondrial control (European×European versus European×Asian, $\chi^2_1=4.71$, $p=0.03$; Asian×European versus Asian×Asian, $\chi^2_1=3.53$, $p=0.06$).

### (c) *No maternal and paternal effects on metabolic rate*

Maternal (or paternal) effects occur when the phenotype of the mother (or father) affects the offspring phenotype in ways additional to the additive genetic effects, and can be either genetically or environmentally determined, the latter for example through maternal investments in eggs. We tested for a maternal genetic effect in analyses of the mitochondrial control hypothesis. Because visual inspection of results (figure 1) suggested a possible paternal effect, we decided also to test the paternal genetic and environmental effects, maternal environmental effects and permanent environmental effects. None of these effects were significant (table 3).

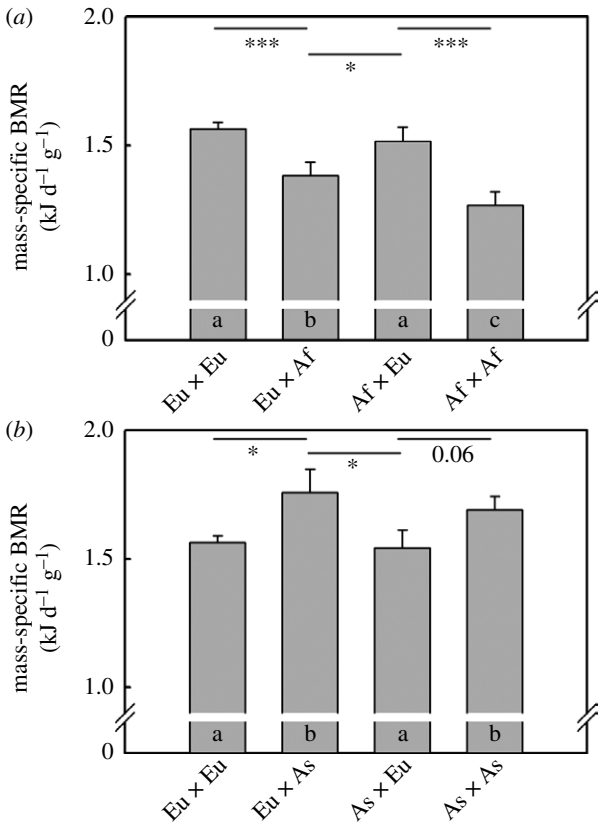


Figure 1. Mass-specific BMR (kJ d$^{-1}$ g$^{-1}$) of stonechats with different parental configurations, specified as mother×father. Origin is abbreviated as Eu, Europe; Af, Africa; As, Asia. Bars are s.e.; for sample sizes see table 2. Identical letters indicate non-significant differences between groups, with a *post hoc* test. *p*-values for pairwise comparisons: $^{***}p<0.0001$, $^{*}p<0.05$. (*a*) European–African pairings; (*b*) Asian–European pairings.

Table 3. Results of animal model analyses to test for maternal, paternal and permanent environmental effects on mass-specific BMR of a panmictic population of stonechats, with individuals from populations in Europe, Africa and Asia, and from crosses among these populations. Estimates of additive genetic variance ($V_A$), variance of effect ($V_E$) and residual variance ($V_R$) are reported ±s.e. Effects were tested using the $\chi^2$-distributed difference between the $-2\times$log likelihood of a model containing the effect and that of the simple model with no effects included.

| model | log likelihood | $V_A$ (s.e.) | $V_E$ (s.e.) | $V_R$ (s.e.) | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|---|
| no effects | 185.199 | 0.0348 (0.0077) | — | 0.0154 (0.0029) | — | — |
| maternal genetic | 185.199 | 0.0348 (0.0077) | 0.0000 (0.0000) | 0.0154 (0.0029) | 0 | 1 |
| maternal environmental | 185.199 | 0.0348 (0.0077) | 0.0000 (0.0000) | 0.0154 (0.0029) | 0 | 1 |
| paternal genetic | 185.373 | 0.0322 (0.0089) | 0.0031 (0.0061) | 0.0154 (0.0029) | 0.348 | 0.56 |
| paternal environmental | 185.225 | 0.0340 (0.0087) | 0.0009 (0.0048) | 0.0153 (0.0029) | 0.052 | 0.82 |
| permanent environmental | 185.367 | 0.0296 (0.0117) | 0.0044 (0.0081) | 0.0149 (0.0029) | 0.336 | 0.56 |

Table 4. Numbers of codons, synonymous (S) and non-synonymous (NS) substitutions, neutrality index (NI) values and *p* values for MK tests for genes, which make up metabolic complexes of mitochondrial subunits. 'Combined' refers to a dataset consisting of all genes combined across all complexes. See appendix 1 in the electronic supplementary material for results per individual gene. Poly, polymorphic substitutions within populations; fixed, fixed substitutions between populations.

| | codons | fixed S | poly S | fixed NS | poly NS | NI | *p* (MK) |
|---|---|---|---|---|---|---|---|
| complex I | 2121 | 266 | 18 | 35 | 6 | 2.53 | 0.099 |
| complex III | 380 | 35 | 38 | 4 | 11 | 2.53 | 0.161 |
| complex IV | 1001 | 103 | 6 | 4 | 1 | 4.29 | 0.275 |
| complex V | 282 | 41 | 5 | 5 | 0 | 0.00 | 1.000 |
| combined | 3784 | 445 | 67 | 48 | 18 | 2.49 | 0.005 |

### (d) *Mitochondrial DNA sequences differ between European and African populations*

Comparing the mitochondrial DNA of European and African stonechats, we found substantial numbers of amino acid-altering substitutions in genes belonging to each complex, with values ranging from 4 amino acid-altering substitutions for genes in complexes III and IV to 35 for genes in complex I (table 4). Results from the MK tests and values for the NI index indicate that, overall, purifying selection acts on amino acid-altering mutations in stonechat mitochondrial DNA, although our small sample size limits the power of our analysis, particularly for MK tests on individual genes (table 4; appendix 1 in the electronic supplementary material). It is also difficult to determine which mitochondrial gene(s) is/are the direct target(s) of selection based on the gene-by-gene tests (cf. appendix 1 in the electronic supplementary material) owing to the linkage between genes in the non-recombining mitochondrial DNA genome.

## 4. DISCUSSION

Hybrids of stonechats from contrasting parental configurations ($female_{high}$–$male_{low}$ versus $female_{low}$–$male_{high}$) differed in metabolic rate, verified in two separate datasets with crosses of high and low metabolism populations. These findings provide evidence that the genetic match between mitochondrial DNA and nuclear DNA has consequences for metabolic rate in birds. Hybrids with contrasting parental configurations have on average identical mixtures of nuclear DNA, and differ only in the mitochondrial DNA that they inherited from the mother. Therefore, our findings implicate mitochondrial function as an important regulator of energy metabolism in birds, and are consistent with the mitochondrial–nuclear coadaptation hypothesis. The mitochondrial control hypothesis, with the *a priori* prediction of a maternal effect, and the nuclear control hypothesis, predicting no differences between hybrids with reciprocal parental configurations, received no support. In combination with the substantial heritabilities of metabolic rate, and corroborated by differences in mitochondrial DNA sequences, these results set the stage for further investigations of the genetic control mechanism determining metabolic rate.

Intergenomic coadaptation between mitochondrial and nuclear DNA, as inferred from our results, has not previously been shown to affect metabolic rate at the whole-organism level in endotherms. Studies on copepods (*Tigriopus californicus*), however, have shown that inter-population hybrids, with mismatched mitochondrial and nuclear genomes, have reduced mitochondrial function resulting from the loss of activity of those complexes in the electron transport chain that involve both mitochondrial and nuclear subunits (Ellison & Burton 2006). In these copepods, impaired mitochondrial function negatively affected fitness measured by hatching number, survival and metamorphosis. Studies on fitness consequences for avian hybrids show ambiguous findings, with some indicating effects on migration and breeding, but others not (Avise & Nelson 1989; Helbig *et al.* 1994; Veen *et al.* 2007). This ambiguous pattern might be explained if the nature and magnitude of mismatch between mitochondrial and nuclear DNA varies and affects the functional or evolutionary significance in different ways.

Our study was inspired by the possibility that a clear set of candidate genes could affect metabolic rate through mitochondrial function, but our results, especially the resemblance between the hybrids' metabolic rate and that of their father (figure 1), could also be explained by alternative mechanisms. Whereas the animal model

analyses do not support paternal and maternal genetic or environmental effects, the sample size is modest and as a result the power of these tests is limited. Alternative explanations fall into three main categories: *control by sex chromosomes*; *genomic imprinting*; and *non-genetic maternal or paternal effects*. *Control by sex chromosomes* in birds could lead to a sex-specific effect on metabolic rate, with sons resembling fathers, both possessing two Z chromosomes, and daughters resembling mothers, both possessing a set of WZ chromosomes. We find this an unlikely explanation for our results because, although sex has a significant effect in our analyses, in most bird species metabolic rate does not differ among sexes. In our stonechats, the direction of the sex effect differs among populations, with females having a higher mass-specific BMR in the European and African populations, and a lower value in the Asian population, when compared with their male counterparts. *Genomic imprinting* (Wilkins & Haig 2003; Keverne 2007), the process whereby the gene inherited from one parent dominates over its counterpart from the other parent, can occur on sets of autosomal chromosomes in both female and male offspring or on the set of Z chromosomes in male offspring. Applying these mechanisms to the results in this paper, the paternally inherited genes involved in metabolic rate could dominate over their maternally inherited counterparts, leading to a paternal effect on metabolic rate. Evidence for genomic imprinting in birds is growing, but there are no studies available that point to effects of genomic imprinting on metabolic rate. Finally, *non-genetic maternal or paternal effects* are possible through a variety of unexplored pathways and cannot be excluded, but no obvious mechanism for such effects in these birds is known at present.

The importance of the genetic match between mitochondrial DNA and nuclear DNA in regulating metabolic rate in hybrid stonechats supplies further evidence that mitochondrial DNA is subjected to selection (e.g. Ballard & Rand 2005; Zink 2005). Our results imply that a coevolutionary response of mitochondrial and nuclear DNA is involved in the selection on mass-specific metabolic rate (see also Blier *et al.* 2001). This connects with studies that support the 'thermal adaptation' hypothesis that climate can be a selective agent that alters mitochondria haplotype, by changing mitochondria function (i.e. ATP production) and heat production (Matsuura *et al.* 1993; Mishmar *et al.* 2003; Ruiz-Pesini *et al.* 2004; Ballard & Rand 2005; Fontanillas *et al.* 2005; Zink 2005). Under the thermal adaptation hypothesis, organisms living in different thermal environments may be under strong selection through the impact that amino acid-altering substitutions in mitochondrial DNA have on heat production.

Estimates of $h^2$ for mass, whole-organism BMR and mass-specific BMR, and of $r_A$ between these traits, are of similar magnitude when based on the current dataset (table 1), which combines intra- and interpopulation variations by the inclusion of hybrids, and on each population separately (B. I. Tieleman, M. A. Versteegh, B. Helm & N. J. Dingemanse 2006, unpublished data). This potentially indicates that within- and among-population variations are largely caused by the same genetic mechanisms. We recognize that our finding of weak genetic correlations between mass, whole-organism BMR and mass-specific BMR is counter-intuitive (table 1), especially in light of the generally significant effects of mass on BMR at the phenotypic level. However counter-intuitive these results might seem, the data indicate that, to a large extent, different suites of genes influence each trait.

In conclusion, we have shown that whole-organism and mass-specific BMRs in stonechats are under genetic control. As implied by the quantitative genetics analyses, metabolism is probably under the influence of multiple genes. The results of the hybrids of reciprocal parental configurations demonstrate that mitochondrial–nuclear coadaptation is at least partly responsible for regulating mass-specific BMR.

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