RESEARCH ARTICLE

Annual cycles of metabolic rate are genetically determined but can be shifted by phenotypic flexibility

M. A. Versteegh[1,*], B. Helm[2,3], E. Gwinner[2,†] and B. I. Tieleman[1,2]

[1]Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 11103, 9700 CC, Groningen, The Netherlands, [2]Max Planck Institute for Ornithology, Von-der-Tann-Strasse 7, 82346 Andechs, Germany and [3]Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, G12 8QQ, UK

*Author for correspondence (m.a.versteegh@rug.nl)
†Deceased

SUMMARY

Birds have adjusted their life history and physiological traits to the characteristics of the seasonally changing environments they inhabit. Annual cycles in physiology can result from phenotypic flexibility or from variation in its genetic basis. A key physiological trait that shows seasonal variation is basal metabolic rate (BMR). We studied genetic and phenotypic variation in the annual cycles of body mass, BMR and mass-specific BMR in three stonechat subspecies (*Saxicola torquata*) originating from environments that differ in seasonality, and in two hybrid lines. Birds were kept in a common garden set-up, under annually variable day length and at constant temperature. We also studied whether stonechats use the proximate environmental factor temperature as a cue for changes in metabolic rate, by keeping birds at two different temperature regimes. We found that the different subspecies kept in a common environment had different annual cycles of body mass, BMR (variance: Kazakh 4.12, European 1.31, Kenyans 1.25) and mass-specific BMR (variance: Kazakh 0.042, European 0.003, Kenyans 0.013). Annual variation in metabolic measures of hybrids was intermediate or similar to that of parental species. Temperature treatment did not affect the shape of the annual cycles of metabolic rate, but metabolic rate was higher in birds kept under the variable temperature regime. The distinct annual cycles in body mass and metabolic rate in stonechat subspecies kept in a common environment indicate different genetic backgrounds rather than merely a phenotypically flexible response to proximate environmental cues. Phenotypic effects of temperature are superimposed on this genetically orchestrated annual cycle.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/19/3459/DC1

Key words: annual cycle, metabolic rate, temperature, common garden, birds.

Received 10 April 2012; Accepted 19 June 2012

INTRODUCTION

Seasonal changes in the environment influence the metabolic rate of birds (McKechnie, 2008; Swanson, 2010) and have repercussions for their behavior, physiology and ecology. Seasonal variation in metabolic rate has been widely studied (for a review, see McKechnie, 2008), often between two seasons (e.g. Ambrose and Bradshaw, 1988; Cooper and Swanson, 1994; Kvist and Lindström, 2001; Wikelski et al., 2003), but sometimes in more detail year round (Piersma et al., 1995; Klaassen, 1995; Zheng et al., 2008; Vézina et al., 2011). The variety of patterns emerging from these studies indicates that annual cycles in metabolic rate differ among environments and/or with life history strategies. For example, resident birds in temperate zones generally have a higher metabolic rate in winter than in summer (Cooper and Swanson, 1994; Cooper, 2000; Dawson, 2003). Temperate or arctic breeding migrants, in contrast, generally have a lower metabolic rate in winter than in summer (Kersten et al., 1998; Kvist and Lindström, 2001; Wikelski et al., 2003). Patterns of resident subtropical, tropical and desert birds are inconsistent, showing no annual variation (Bush et al., 2008; Maldonado et al., 2009; Doucette and Geiser, 2008), an increase (Chamane and Downs, 2009) or a decrease in metabolic rate in winter compared with summer (Maddocks and Geiser, 2000; Smit and McKechnie, 2010). Whether variation in annual cycles in

metabolic rate found in birds results from genetic differences or phenotypic flexibility is largely unknown.

To compare the seasonality of energy metabolism, researchers use different measures of metabolic rate, including basal metabolic rate (BMR), resting metabolic rate or field metabolic rate. BMR is the most standardized measure, defined as the minimum energy expenditure of a post-absorptive normothermic animal, measured during the rest phase, at thermoneutral temperature (King, 1974). Mass-specific BMR, calculated as BMR divided by body mass (in $kJ\,day^{-1}\,g^{-1}$), is commonly applied in studies evaluating tissue-level processes, such as mitochondrial function, production of free radicals and other measures related to aging or intrinsic mortality (e.g. Speakman, 2005; Tieleman et al., 2009b). BMR's relationship to activity (Deerenberg et al., 1998; Nudds and Bryant, 2001), food availability and diet (McNab, 1988; Mueller and Diamond, 2001), organ size and body composition (Daan et al., 1990; Piersma et al., 1996; Tieleman et al., 2003), and daily energy expenditure (Daan et al., 1990; Nilsson, 2002; Tieleman et al., 2008) makes it an interesting trait for researchers studying seasonal variation.

BMR is influenced by seasonally variable environmental factors such as temperature and food availability (Swanson and Olmstead, 1999; Tieleman et al., 2003; Broggi et al., 2007; Swanson, 2010), but also by seasonally variable life cycle traits and physiology

(Klaassen, 1995; Ricklefs and Wikelski, 2002; Wikelski et al., 2003; Vézina et al., 2007). There are several hypotheses about the mechanisms underlying the adjustment of BMR to the annual cycle of energetic needs and challenges. Annual cycles of BMR could have a genetic basis, indicating that annual cycles in metabolism have evolved to be adaptive in particular environments and not in others (Piersma and Drent, 2003; Hahn and MacDougall-Shackleton, 2008). Alternatively, or superimposed on a genetically orchestrated pattern, annual cycles in BMR could result from phenotypically flexible adjustments (Piersma and Drent, 2003; Hahn and MacDougall-Shackleton, 2008). This would allow birds to adjust their cycles in BMR at short notice to the needs of any environmental conditions that they encounter. Whether annual cycles in metabolic rate have a genetic basis or result solely from phenotypic flexibility can be investigated by placing species from seasonally different environments in a common environment (Turesson, 1922; Gwinner et al., 1995a). If the annual cycles have a genetic basis, differences among species will be maintained. However, if annual cycles of metabolism result solely from phenotypic flexibility, birds will adjust their metabolism to the common environment and no difference will be detected among the species. Additional means to investigate genetic and phenotypically flexible components of annual cycles include studying hybrids created from birds with different annual cycles, or altering environmental conditions at which birds are kept.

Stonechats (*Saxicola torquata*) provide an ideal study system to address the evolutionary physiology of annual cycles in metabolic rate because we can build on extensive knowledge from previous studies of their physiology and life history. Stonechats, widespread small passerines (Urquhart, 2002), occur in environments that vary considerably in seasonal cycles of environmental conditions, which is reflected in distinct annual cycles and life history traits (Gwinner et al., 1995a; König and Gwinner, 1995; Helm et al., 2009; Helm, 2009). They are easily kept and bred in captivity, and hybrid lines between subspecies can be created. Differences in life history traits among stonechat subspecies remain present when birds are born and held in captivity (Gwinner et al., 1995a; König and Gwinner, 1995; Helm et al., 2009; Helm, 2009). The mass-specific metabolic rate of birds kept at the same temperature and the same or varying light regimes was lowest for Kenyan stonechats, intermediate for European stonechats and highest for Kazakh stonechats (Klaassen, 1995; Wikelski et al., 2003; Tieleman, 2007). In addition, repeated measures, inclusion of pedigree information and hybridization among subspecies have revealed that BMR in stonechats has a repeatable, heritable component (Versteegh et al., 2008; Tieleman, et al., 2009a; Tieleman et al., 2009b). A logical extension of these stonechat studies is to investigate whether annual cycles in metabolism differ among birds from different environments, and if differences in annual cycles have a genetic basis or result solely from phenotypic responses to annual environmental and life cycle variation.

We studied the genetic and phenotypic backgrounds of annual cycles in body mass, BMR and mass-specific BMR of Kazakh, European and Kenyan stonechats, and their hybrids, bred and held in a common environment. We compared variability (i.e. differences between life cycle stages with the highest and the lowest value) and also shape (i.e. the particular pattern of upregulation and downregulation) of annual cycles between subspecies. The rationale is that the subspecies can upregulate or downregulate traits (i.e. BMR) in different life cycle stages, resulting in different shapes, or display similar shapes but differ in variability (e.g. Gwinner et al., 1995b). If annual cycles in body mass and metabolic rate have a genetic background, we would expect the shape and/or variability

to differ among subspecies, and the hybrid lines to show values intermediate between their parent groups. To investigate whether phenotypic flexibility has an (additional) effect on annual cycles, we kept European stonechats under two different temperature regimes, either at year-round constant temperature or at annually fluctuating temperature.

MATERIALS AND METHODS
Birds and keeping conditions

Stonechats originated from three different locations: Kazakhstan (*N*=17) [*S. t. maurus* (Pallas 1773)], Europe (*N*=61) [*S. t. rubicola* (Linnaeus 1766)] and Kenya (*N*=22) [*S. t. axillaris* (Shelley 1884)]. Kazakh stonechats migrate over long distances and have a short breeding season during which they typically lay a single clutch with five to six eggs; European stonechats are short-distance migrants that typically lay two to three clutches with five eggs per clutch; Kenyan stonechats are resident birds and lay generally one clutch with three eggs (Helm et al., 2006; Urquhart, 2002; Flinks and Pfeifer, 1987; Helm, 2009). Birds were from the first (*N*=30), second (*N*=48) or third (*N*=28) generation, bred and hand-raised at the Max Planck Institute for Ornithology, Andechs, Germany, or had been taken as nestlings from the field, moved to the institute and hand-raised (*N*=32) (Gwinner et al., 1987). In addition to the three subspecies, we bred and studied hybrids between Kazakh and European subspecies (*N*=17) and between European and Kenyan subspecies (*N*=21). All individuals were fully grown, ranging in age from 0 (age >158 days) to 12 years, but most individuals were 0–4 years old (*N*=128). To investigate whether birds of age 0 had reached adult-level metabolic rates, we tested whether they differed significantly from older birds (aged 1–12 years) in body mass, BMR and mass-specific BMR; they did not ($F_{1,237}$<2.59, P<0.11). Therefore, we concluded that birds had reached adult-level metabolic rates.

Birds were housed with 8–12 individuals per room and individuals were kept in separate cages. They were randomly assigned to rooms with respect to subspecies. Standard conditions in all rooms consisted of year-round constant temperatures of 20–22°C and day length following natural day length of the European population (see Helm et al., 2009). In addition to this standard treatment, we also kept a subset of European birds (*N*=14) under a weekly changing temperature regime that mimicked the average natural temperature cycles of free-living European stonechats reconstructed from ringing recovery locations (Helm et al., 2006).

Measurements

We performed measurements during five life cycle stages, between February 2005 and March 2006: the spring migration period (27 February to 24 March), breeding period (10 May to 2 June), molt (21 July to 19 August), autumn migration period (30 September to 7 November) and winter (24 November to 14 February). During the migration period birds are active during the night (Helm et al., 2005), which we monitored by a constant infrared light beam. Kenyan stonechats show migratory restlessness when held in captivity, even though free-living Kenyan stonechats do not migrate. We defined winter as the quiescent period after the autumn migration period and before the spring migration period. Stonechats were unpaired during the breeding season, but they do physiologically prepare for breeding after night-time activity (due to spring migration) stops, and before molt starts (Gwinner et al., 1995b; Helm et al., 2005). Birds were checked twice a week for molt. One hour before the start of experiments, we removed water and food from cages to ensure birds were post-absorptive. We placed birds in 13.51

metal metabolic chambers with Plexiglas lids that made an airtight seal. We set the metabolic chambers inside a climatic chamber with a constant temperature of 35±0.5°C, which is within the thermoneutral zone of the stonechat subspecies (Tieleman, 2007).

We measured body mass and BMR of Kazakh, European and Kenyan stonechats and the two hybrid lines. Metabolic rate was measured by standard flow-through respirometry methods (Gessaman, 1987). Details about experimental set up, measurement protocol and data analysis are described elsewhere (Tieleman, 2007; Versteegh et al., 2008; Tieleman et al., 2009a). To ensure that birds were not active, we only used measurements obtained at least 3h after birds were put in the metabolic chambers, and only when O_2 consumption had been stable for at least 10min. Before and after the metabolic measurements were taken, we measured body mass and calculated mass-specific BMR by dividing BMR by the mean of the two body masses. We normally measured 6–17 individuals per life cycle stage per group kept at standard conditions. Because we combined this with other studies (Versteegh et al., 2008; Tieleman et al., 2009a) some sample sizes for winter are larger (Europeans N=45, European × Kenyan hybrids N=21). We measured 7–13 individuals per life cycle stage of European stonechats kept at variable temperature.

Statistical analysis

We used R version 2.8.0 for statistical analyses (R Development Core Team, 2010). Variables were normally distributed (Kolmogorov–Smirnov D<0.09, P>0.10), and we used mixed effects models for body mass, BMR and mass-specific BMR. We also analyzed BMR with body mass as covariate, but we do not report the results because they did not differ from the analyses of mass-specific BMR. In all mixed effects models we used log-likelihood ratio tests and the χ^2-statistic to evaluate statistical significance (P<0.05). We investigated whether the annual cycles of body mass, BMR and mass-specific BMR of different subspecies (including hybrid lines) or treatment groups differed in shape (given by interactive effects of subspecies and life cycle stage) and/or variability (given by variance, see below). We first analyzed variation among life cycle stages and subspecies in birds kept at standard conditions with models including subspecies, life cycle stage, sex, age and the interactions life cycle stage × subspecies, life cycle stage × sex and subspecies × sex. We analyzed the response to temperature treatment in European stonechats with models including treatment, life cycle stage, sex, age and the interactions life cycle stage × treatment, life cycle stage × sex and subspecies × sex. Because we measured the same individuals during multiple life cycle stages, we included individual as a random effect in all models.

If the interaction life cycle stage × subspecies or life cycle stage × treatment was significant, we tested the significance of life cycle stage in each subspecies or treatment group separately. If life cycle stage was significant in these separate analyses, we subsequently used a *post hoc* Tukey test to compare shape and variability of annual cycles in body mass, BMR and mass-specific BMR. Additionally, we calculated the means per life cycle stage for each subspecies or treatment group separately. To investigate differences in variability of the traits, we calculated annual means and variances using these life cycle stage means. We compared the variances in a pair-wise fashion among subspecies, with a one-tailed F-test. We first compared annual cycles of Kazakh, European and Kenyan subspecies, to investigate whether they are based on genetic or solely on phenotypic differences. To further investigate genetic components of annual cycles, we then compared hybrid stonechats (Kazakh × European or European × Kenyan) with their parental subspecies. We report means of body mass, BMR and mass-specific BMR per subspecies based on models without the interaction terms life cycle stage × subspecies. We visualized the results of statistical analyses on the subspecies by plotting residuals from models including subspecies as a fixed effect.

RESULTS
Subspecies differences in annual cycles of mass and metabolism

Confirming previous work, mass-specific BMR was highest in Kazakh, intermediate in European and lowest in Kenyan stonechats, and body mass was lowest in Kazakh and highest in Kenyan stonechats (Table 1). Annual cycles in body mass, BMR and mass-specific BMR differed in shape (χ^2>38.59, d.f.=16, P<0.001; Table 2A, Fig. 1) as well as in variability (Table 1) among subspecies and hybrid lines of stonechats kept in a common environment. Male stonechats were significantly heavier and had a lower mass-specific BMR than females (Table 2A).

Comparing subspecies in a common environment

The shape of annual cycles of body mass was similar in Kazakh and Kenyan stonechats, but Europeans deviated, especially in the breeding season (Fig. 1A; supplementary material Table S1). Relative to the subspecies' average, Kazakh and Kenyan birds were especially heavy in the two migration periods, and lighter during breeding, molt and winter. European stonechats were also heavier in the migration periods, and lighter during molt and winter, but differences among these life cycle stages were less pronounced and not significant (supplementary material Table S1). However, in the breeding period, Europeans were relatively heavy, unlike Kazakh and Kenyan stonechats. Despite the impression of a smaller seasonal variation in the European birds, the variability did not significantly differ among subspecies (Table 1).

Annual cycles of BMR differed in shape among subspecies (Fig. 1B; supplementary material Table S1). Kazakh stonechats had a relatively high BMR during breeding and a low BMR during the

Table 1. Variance of the means per life cycle stage and overall subspecies means ± s.e.m. for body mass, BMR and mass-specific BMR of Kazakh, European and Kenyan stonechats and two hybrid lines

	Body mass (g)		BMR (kJ day^{-1})		Mass-specific BMR (kJ day^{-1} g^{-1})	
	Variance of life cycle stage means	Subspecies mean ± s.e.m.	Variance of life cycle stage means	Subspecies mean ± s.e.m.	Variance of life cycle stage means	Subspecies mean ± s.e.m.
Kazakh	1.636[a]	13.8±0.56[a]	4.120[a]	22.4±0.73[a]	0.042[a]	1.63±0.06[a]
Kazakh × European	1.104[a,b]	14.4±0.40[a,b]	0.567[b]	23.1±0.51[a]	0.019[a,b]	1.63±0.04[a]
European	0.474[a,b]	14.3±0.47[a]	1.311[a,b]	22.0±0.60[a]	0.003[b]	1.54±0.05[a,b]
European × Kenyan	0.184[b]	15.6±0.52[b]	0.568[b]	22.1±0.67[a]	0.006[a,b]	1.43±0.06[b]
Kenyan	0.967[a,b]	17.7±0.58[c]	1.249[a,b]	22.3±0.74[a]	0.013[a,b]	1.26±0.06[c]

Different letters indicate significant differences (F-test, P<0.05).

Table 2. Degrees of freedom, χ^2- and *P*-values of log-likelihood ratio tests for body mass, BMR and mass-specific BMR of stonechats kept at constant and variable temperature

	d.f.	Body mass		BMR		Mass-specific BMR	
		χ^2	P	χ^2	P	χ^2	P
A							
Life cycle stage × subspecies	16	38.59	**0.001**	48.71	**<0.001**	48.89	**<0.001**
Subspecies × sex	4	1.66	0.80	4.04	0.40	4.11	0.39
Life cycle stage × sex	4	3.24	0.52	1.72	0.79	1.69	0.79
Subspecies	4	–	–	–	–	–	–
Life cycle stage	4	–	–	–	–	–	–
Sex	1	16.13	**<0.001**	1.73	0.19	5.58	**0.02**
Age	1	0.12	0.73	0.14	0.71	0.93	0.33
B							
Life cycle stage × treatment	4	7.01	0.14	10.26	**0.04**	2.66	0.62
Treatment × sex	1	0.76	0.38	0.35	0.55	0.05	0.82
Life cycle stage × sex	4	5.71	0.22	12.61	**0.01**	4.28	0.37
Treatment	1	0.95	0.33	–	–	6.31	**0.01**
Life cycle stage	4	32.21	**<0.001**	–	–	10.01	**0.04**
Sex	1	15.21	**<0.001**	–	–	6.73	0.01
Age	1	1.12	0.29	0.08	0.78	1.06	0.30

Data are for (A) Kazakh, Kazakh × European, European, European × Kenyan and Kenyan stonechats and (B) European stonechats.
Results are from linear mixed models with individual as random effect, after backwards elimination of non-significant (*P*>0.05) terms. When interactions are significant, statistics of main effects cannot be meaningfully interpreted (Looney and Stanley, 1989), and therefore we do not show these.
Significant *P*-values are in bold.

autumn migration period. In contrast, in European stonechats BMR was not elevated during breeding, and was relatively low during both molt and the autumn migration period. Contrary to both Kazakh and European birds, BMR of Kenyan stonechats was especially elevated during molt. Although the Kazakh birds appeared to have the largest variation among life cycle stages, the variability did not significantly differ among subspecies (Table 1).

The shape of annual cycles of mass-specific BMR differed among subspecies (Fig. 1C; supplementary material Table S1). Kazakh stonechats had low values during spring and especially the autumn migration period, and high values in the breeding period. European stonechats showed little variation in mass-specific BMR throughout the year. In Kenyan stonechats mass-specific BMR was elevated during molt and reduced during the autumn migration period. The other life cycle stages did not differ from each other. The variability was significantly different among subspecies: Kazakh had higher variance than European stonechats ($F_{4,4}$=12.37, *P*=0.02; Table 1), while Kenyan stonechats were intermediate.

Comparing hybrids with parent lines

Body mass, BMR and mass-specific BMR of hybrids showed intermediate values to parental subspecies in some seasons, and in other seasons resembled one of the parent lines (Fig. 1, Tables 1, 2). The body mass of Kazakh × European stonechats showed an annual cycle similar in shape to that of Kazakh stonechats, but it lacked the high values in the spring migration period and showed a larger dip during molt, more closely resembling European birds (Fig. 1A). There was no significant difference in body mass variability among Kazakh × European hybrids and their parental subspecies (Table 1). Body mass of European × Kenyan hybrids was intermediate to both parent groups during breeding, the autumn migration period and winter. The European × Kenyan hybrids showed an increase in body mass from molt to the autumn migration period, similar in shape but smaller in magnitude compared with that of Kenyan stonechats (Fig. 1A). The variability was not intermediate and appeared even to be lower in the European × Kenyan hybrids than in either of the parental subspecies (Table 1).

The shape of the annual cycle in BMR of Kazakh × European hybrids was similar to that of European stonechats, lacking the pronounced high and low values during breeding and the autumn migration period, respectively, of the Kazakh birds (Fig. 1B; supplementary material Table S1). The variability among life cycle stages of BMR was significantly lower in Kazakh × European hybrids than in Kazakh stonechats ($F_{4,4}$=7.27, *P*=0.04; Table 2). In European × Kenyan hybrids BMR resembled European stonechats during the spring migration period, Kenyan stonechats during breeding and the autumn migration period, and was intermediate between parent lines during molt (Fig. 1B). The variability was not significantly different between European × Kenyan stonechats and their parental subspecies (Table 1).

Mass-specific BMR of Kazakh × European hybrids was similar to that of Kazakh birds during breeding, molt and the autumn migration period, but close to values of European stonechats during the spring migration period and winter (Fig. 1C). Mass-specific BMR of Kazakh × European hybrids did not differ significantly in variability from either Kazakh or European stonechats. The fact that the two parental subspecies did differ from each other implies that annual variation of this hybrid line is intermediate to that of its parental subspecies (Table 1). In European × Kenyan hybrids mass-specific BMR was high during molt and low during the autumn migration period, similar in shape to the Kenyan stonechats (Fig. 1C). During the spring migration period, breeding and winter mass-specific BMR varied little in either European × Kenyan hybrids or their parental subspecies. The variability of mass-specific BMR of European × Kenyan hybrids did not differ from that of European and Kenyan stonechats (Table 1).

Phenotypic flexibility of annual cycles of mass and metabolism: response to temperature

We found that the shape and variability of the annual cycles of body mass and mass-specific BMR did not differ between European stonechats exposed to different temperature treatments (Fig. 2B,D, Table 2B, Table 3). After removing the non-significant interaction term treatment × life cycle stage, we found that mass-specific BMR
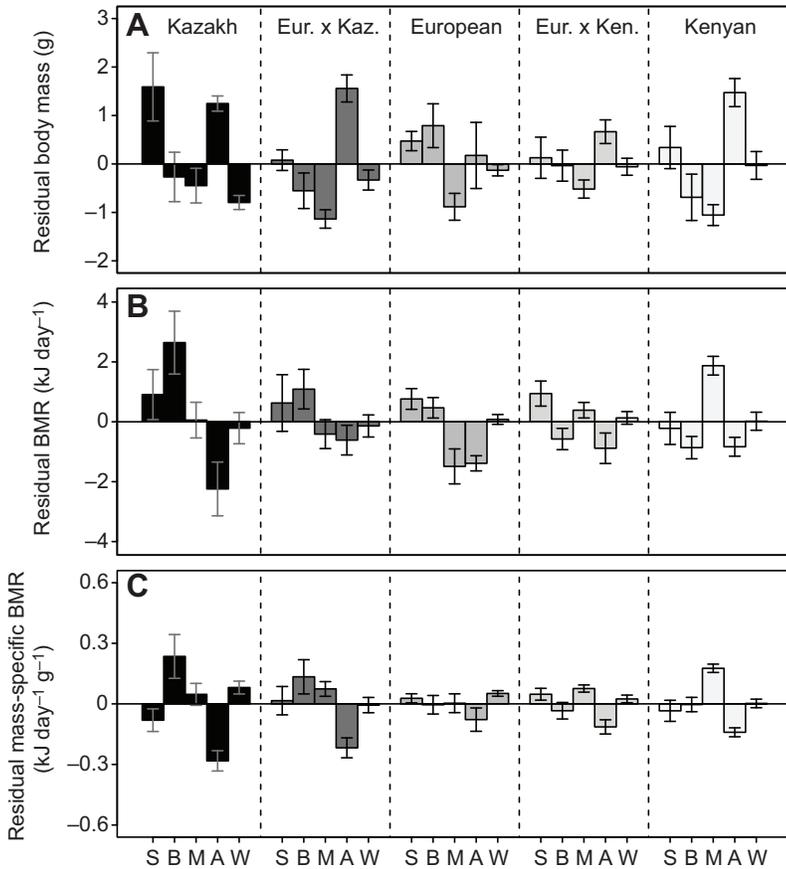
Fig. 1. Annual variation of (A) body mass, (B) basal metabolic rate (BMR) and (C) mass-specific BMR of Kazakh, European and Kenyan stonechats, and Kazakh × European and European × Kenyan hybrid lines. Stonechats were kept at constant temperature and annually fluctuating day length. Bars represent means ± s.e.m. of residuals of a linear model with subspecies as fixed effect. Letters represent life cycle stages: S, spring migration period; B, breeding season; M, molt; A, autumn migration period; and W, winter.

differed between treatment groups, whereas body mass did not (Table 2B). The effect of life cycle stage was significant for both body mass and mass-specific BMR (Table 2B). Taking the significant term life cycle stage into account, stonechats kept at variable temperature had an 8% higher mass-specific BMR than birds kept at constant temperature (Fig. 2D).

For BMR, the annual cycles differed between treatment groups, as indicated by the significant interaction treatment × life cycle stage (Table 2B, Fig. 2C). We first compared the shape of the annual cycles using *post hoc* Tukey tests comparing life cycle stages with each other per treatment, and found that the difference occurred in the autumn migration period. During the autumn migration period, stonechats kept at variable temperature had a BMR close to the treatment group's average, while stonechats kept at constant temperature showed a relatively low value (supplementary material Table S1). The variances of BMR did not differ among treatment groups (Table 3). Because different temperature regimes can also result in overall differences in BMR between groups in some or all life cycle stages, we also explored for each life cycle stage whether treatment groups differed from each other. During the spring migration period (χ^2=3.87, d.f.=1, P=0.049) and the autumn migration period (χ^2=12.49, d.f.=1, P<0.001) stonechats kept at constant temperature had a significantly lower BMR than stonechats kept at variable temperature. In the other life cycle stages the treatment groups did not differ from each other (χ^2<2.21, d.f.=1, P>0.14).

DISCUSSION

Three subspecies of stonechats originating from environments that differ in seasonality maintained different annual cycles in body mass, BMR and mass-specific BMR, when reared and kept in a common

environment in captivity. In addition, two hybrid lines bred between subspecies displayed different annual cycles, with values often intermediate to the parent lines. Keeping stonechats at two temperature regimes, one constant and one variable throughout the year, did not affect the shape or variability of the annual cycles of body mass and mass-specific BMR. However, mass-specific BMR was 8% higher throughout the year in the birds kept at variable temperature, and BMR was higher in birds kept at variable temperature in two life cycle stages. These findings show that variation in the shape and variability of annual cycles in body mass and metabolic measures stems from genetic differences, and that phenotypic flexibility contributes modest variation superimposed on the genetic program.

The genetic background of the differences in annual cycles among Kazakh, European and Kenyan stonechats suggests evolutionary modification. Evolution of annual cycles in body mass or metabolic rate could be a direct response to the changing environmental conditions that different subspecies encounter throughout the year (e.g. food availability, pathogen and predation risk, temperature), but could also be connected with variation in other life history characteristics (Daan et al., 1990; Ricklefs and Wikelski, 2002). One might expect more variation in the annual cycle of birds that encounter larger environmental differences during the year or that vary their work levels more during the year. Because it is challenging to provide the bird's perspective on the environmental conditions encountered, especially by migrant birds, we first explored whether life history differences could explain the annual cycle differences among stonechats. Because Kazakh stonechats breed in a continental environment, lay the largest clutch and migrate the longest distance (Raess, 2008), we expected that they would experience the largest differences in energy expenditure among life cycle stages. We
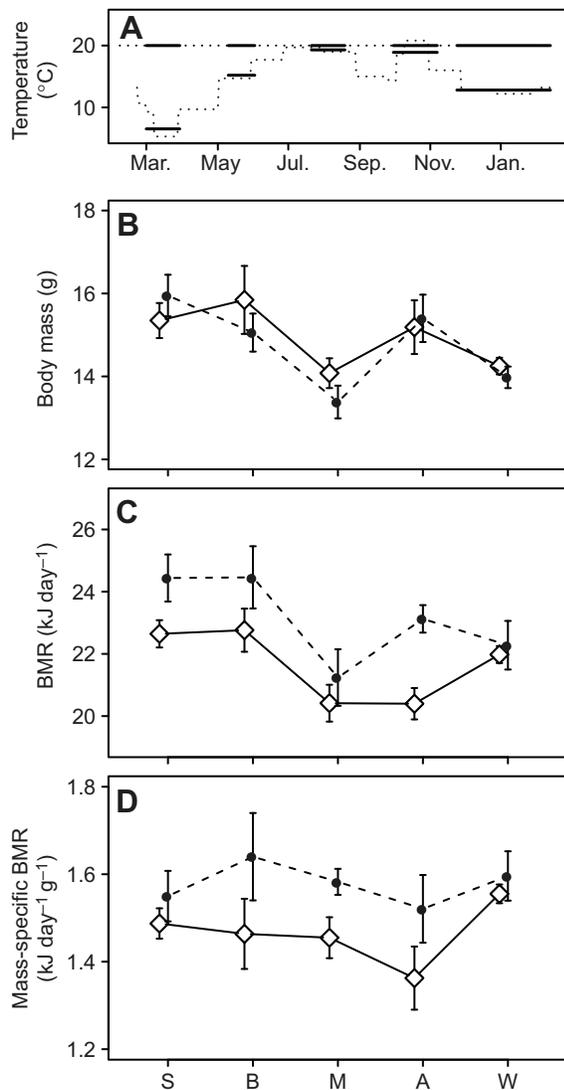
Fig. 2. (A) Annual variation in temperature in constant and variable temperature treatment groups. The dotted line represents the temperature birds were exposed to, the solid lines the measurement periods. (B) Body mass, (C) BMR and (D) mass-specific BMR of European stonechats kept at constant (diamonds) and variable (circles) temperature and measured during five life cycle stages. Symbols represent means ± s.e.m. Letters represent life cycle stages: S, spring migration period; B, breeding season; M, molt; A, autumn migration period; and W, winter.

small clutch per year (Dittami and Gwinner, 1985). Therefore, if the evolution of body mass and metabolic measures is mainly influenced by life history traits, we would expect the subspecies to have evolved in such a way that Kenyan stonechats show the least, European stonechats show intermediate and Kazakh stonechats show the most variability because of their respective migratory and reproductive characteristics. In agreement with these ideas, long-distance migrant Kazakh stonechats indeed had the highest annual variability in all measures. However, in contrast to predictions, European and not Kenyan stonechats showed the lowest variability in mass-specific BMR. We conclude that differences in variability among subspecies are hard to generalize, but subspecies differ substantially in specific life cycle stages, such as migration and molt. This may indicate that, in addition to specific life cycle stage demands, local environmental factors like temperature and food availability also play important roles in the evolution of the annual cycles of mass and metabolic rate.

During the autumn migration period all three stonechat subspecies increased body mass and decreased BMR and mass-specific BMR. During the spring migration period body mass generally increased, BMR increased in Kazakh and European stonechats, and mass-specific BMR decreased in Kazakh stonechats. Increases in body mass just before and during migration periods are commonly found in many captive and free-living bird species, and are related to fat deposition (e.g. Gwinner, 1996; Schaub and Jenni, 2000; Vézina et al., 2007). This could present an explanation for the decrease of mass-specific BMR during the autumn migration, but not for the variation among subspecies in metabolic rate during spring migration. The lower metabolic rate during autumn than during spring migration is also found in free-living migrating yellow-rumped warblers (Swanson and Dean, 1999). In several waders BMR was lower during autumn migration than during winter in the tropics, but higher than during breeding in the arctic (Lindström, 1997; Kvist and Lindström, 2001). BMR depends to a large extent on body and/or organ composition (Hume and Biebach, 1996; Piersma et al., 1999; Dietz et al., 1999; Tieleman et al., 2003). In free-living birds spring migration is often faster (and more urgent) than autumn migration (Pearson and Lack, 1992), and different organs may be important in spring and autumn (e.g. reproductive organs) (see Bauchinger et al., 2005), leading to a difference in BMR in the two migration periods.

BMR and mass-specific BMR of Kenyan stonechats was remarkably high during molt, a finding that was lacking in the other subspecies. Klaassen showed that Kenyan stonechats had a higher total plumage mass than European stonechats (Klaassen, 1995). This is in concordance with the finding of Tieleman that the insulation of Kenyan stonechats was better than that of European and Kazakh stonechats (Tieleman, 2007). Basal metabolic rate has been shown to be correlated with the amount of feathers produced per day (Dietz et al., 1992; Lindström et al., 1993). Therefore, our results support Klaassen's (Klaassen, 1995) hypothesis of a higher increase in BMR between molt and the preceding and following life cycle stages in Kenyan than in European stonechats.

expected European stonechats to be intermediate, because they breed in temperate climates, have an intermediate clutch size, a longer breeding season and a shorter migration distance (Helm, 2009). Free-living Kenyan stonechats may experience the least variation among life cycle stages because they do not migrate and only produce one

Table 3. Variance of means of the five life cycle stages for body mass, BMR and mass-specific BMR of European stonechats kept at variable and constant temperature

	Body mass (g)	BMR (kJ day^{-1})	Mass-specific BMR (kJ day^{-1} g^{-1})
Constant temperature	0.474	1.311	0.003
Variable temperature	1.092	1.775	0.002

No significant differences between variable and constant temperature treatments.

Body mass, BMR and mass-specific BMR of the two hybrid lines were intermediate to values for parental subspecies in some life cycle stages, but also sometimes resembled one of the parental subspecies or deviated from both. This supports a genetic basis for the annual cycles of these traits, but also shows that the underlying genetic mechanisms are not simple. Metabolic rate is determined by the size of the metabolically most active organs, mitochondrial density and mitochondrial function (Daan et al., 1990; Tieleman et al., 2003; Tieleman et al., 2009a; Vézina and Williams, 2005; Zheng et al., 2008), which all may have a genetic basis. Changes in organ size, mitochondrial density or mitochondrial function may contribute to annual changes in metabolic rate (Piersma et al., 1999; Dietz et al., 1999; Tieleman et al., 2003; Zheng et al., 2008). Previous work on stonechat hybrids has shown that metabolic rate is affected, presumably through mitochondrial function, by the mix of mitochondrial genes (inherited only from the mother) and nuclear genes (inherited from both parents), and is not a linear intermediate between parent lines (Tieleman et al., 2009a). Likewise, the inheritance patterns of annual changes in body composition or in mitochondrial density from parental to hybrid stonechats may be complex. Such complicated genetic backgrounds may have resulted in the dissimilar shapes and variabilities that characterized the annual cycles of the two hybrid lines.

The shape and variability of the annual cycles of European stonechats were unaltered by temperature treatments, at least for body mass and mass-specific BMR, and differed only during the migration periods for BMR. Yet, the stonechats kept at variable temperature had a higher mass-specific BMR in all life cycle stages than birds kept at constant temperature. This suggests that European stonechats adjust their metabolic rates to variability, and not so much to a fixed or current temperature. In contrast, red knots (*Calidris canutus*) kept at variable outside temperatures had a BMR similar to warm-acclimated conspecifics in summer and to cold-acclimated conspecifics in winter (Vézina et al., 2011). Also, free-living temperate resident birds are found to adjust their BMR and/or mass-specific BMR according to winter temperatures (Swanson and Olmstead, 1999; Broggi et al., 2007). Vézina et al. proposed that the adjustments in the annual cycle of metabolism of red knots stem from the highly variable ambient conditions (e.g. high and low temperatures) that knots face during the annual cycle (Vézina et al., 2011), a fact that may also apply to temperate resident species (Swanson and Olmstead, 1999; Broggi et al., 2007). Free-living European stonechats may keep their thermal environment relatively constant throughout the year, because they breed in central Europe and winter in southern Europe and northern Africa, where winters are mild (Helm et al., 2006). The necessity of flexibility in the annual cycles of BMR and body mass may be less strong than in birds facing large temperature changes, and therefore annual cycles may be more rigid.

In summary, the variation in annual cycles of body mass, BMR and mass-specific BMR among stonechat subspecies that we found indicates that these cycles have a genetic component. This genetic component is unlikely to be solely an adaptation to the energy demands and costs of life history characteristics. Environmental factors may have contributed to the evolution of annual cycles of BMR as well. The fact that hybrids differ from parent lines confirms that there is a genetic component determining annual cycles of metabolic rate. However, the complexity of the patterns shows that heritability is not simple. When birds were exposed to different temperature regimes, they adjusted their metabolic rate. This shows that, although annual cycles in metabolic rate are genetically determined, annual cycles of metabolic rate are also phenotypically flexible.

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Table S1 Z- and P- values of pair-wise post hoc Tukey tests between life cycle stages (A) per subspecies and hybrid line and (B) per treatment group. The effect of the interaction life cycle stage*treatment was not significant for body mass and mass-specific BMR in European stonechats kept at different temperature treatments. Therefore we did not perform post-hoc tests on these measures for birds kept at fluctuating temperature.

A	Standard temperature									
	Kazakh		Kazakh x European		European		European x Kenyan		Kenyan	
	|z|-value	P	|z|-value	P	|z|-value	P	|z|-value	P	|z|-value	P
Body mass										
spring migration – breeding	4.335	<0.001**	1.418	0.61	0.676	0.96	0.354	1.00	1.807	0.37
breeding – moult	0.062	1.00	1.265	0.71	2.799	0.04*	1.086	0.81	0.645	0.97
moult - autum migration	3.748	0.002**	7.042	<0.001**	1.809	0.36	2.658	0.06	4.444	<0.001**
autumn migration – winter	5.012	<0.001**	5.571	<0.001**	1.244	0.72	1.978	0.27	2.854	0.03
winter - spring migration	5.988	<0.001**	0.959	0.87	2.348	0.13	0.516	0.99	0.631	0.97
spring migration – moult	4.558	<0.001**	2.837	0.04*	2.572	0.07	1.308	0.68	2.452	0.10
breeding - autumn migration	3.304	0.008**	5.11	<0.001**	0.942	0.88	1.572	0.51	3.8	0.001*
moult – winter	0.888	0.90	2.169	0.19	0.988	0.86	1.128	0.79	2.105	0.22
spring migration - autumn migration	1.529	0.54	3.876	0.001**	0.435	0.99	1.027	0.84	1.993	0.27
breeding – winter	0.698	0.96	0.619	0.97	2.524	0.08	0.14	1.00	1.385	0.64
BMR										
spring migration - breeding	1.300	0.69	0.421	0.99	0.642	0.97	2.373	0.12	1.045	0.83
breeding - moult	2.348	0.13	1.472	0.58	2.903	0.03*	1.657	0.45	4.446	<0.001**
moult - autum migration	1.869	0.33	0.302	1.00	0.195	1.00	2.187	0.18	4.397	<0.001**
autumn migration - winter	2.014	0.25	0.589	0.98	2.867	0.03*	2.176	0.18	1.578	0.51
winter - spring migration	1.109	0.80	0.991	0.86	1.288	0.69	1.387	0.63	0.452	0.99

spring migration - moult	0.880	0.90	1.119	0.80	3.998	<0.001**	0.915	0.89	3.401	0.006**
breeding - autumn migration	4.016	<0.001**	1.828	0.36	2.633	0.06	0.530	0.98	0.048	1.00
moult - winter	0.107	1.00	0.224	1.00	3.219	0.01*	0.415	0.99	3.391	0.006**
spring migration - autumn migration	2.486	0.09	1.481	0.57	3.654	0.002**	2.840	0.04*	0.996	0.86
breeding - winter	2.733	0.047*	1.370	0.65	0.326	1.00	1.548	0.53	1.633	0.47
mass-specific BMR										
spring migration - breeding	3.209	0.01*	1.305	0.69	0.653	0.96	1.4920	0.56	0.625	0.97
breeding - moult	2.298	0.14	0.591	0.98	0.078	1.00	2.2420	0.16	3.608	0.003**
moult - autum migration	3.837	0.001**	3.795	0.001**	1.255	0.71	3.8760	< 0.001**	6.368	<0.001**
autumn migration - winter	4.996	<0.001**	3.045	0.02*	3.007	0.02*	3.5380	0.003**	3.252	0.01*
winter - spring migration	1.975	0.27	0.285	1.00	1.264	0.71	0.3230	1.00	0.832	0.92
spring migration - moult	1.210	0.74	0.746	0.94	0.560	0.98	0.4780	0.99	4.232	<0.001**
breeding - autumn migration	5.702	<0.001**	4.093	< 0.001**	1.158	0.77	1.6350	0.47	2.760	0.045*
moult - winter	0.667	0.96	1.118	0.80	1.564	0.51	1.0230	0.84	3.964	<0.001**
spring migration - autumn migration	2.048	0.24	2.910	0.03*	1.967	0.28	2.9300	0.03*	2.136	0.20
breeding - winter	1.985	0.27	1.650	0.46	1.674	0.44	1.6150	0.48	0.124	1.00

Table S1 Continued

B	Temperature treatment			
	Constant		Variable	
	|z|-value	P	|z|-value	P
BMR				
spring migration - breeding	0.642	0.97	0.492	0.99
breeding - moult	2.903	0.03*	2.55	0.08
moult - autum migration	0.195	1.00	1.872	0.33
autumn migration - winter	2.867	0.03*	0.758	0.94
winter - spring migration	1.288	0.69	2.375	0.12
spring migration - moult	3.998	<0.001**	3.644	0.002**
breeding - autumn migration	2.633	0.06	0.745	0.94
moult - winter	3.219	0.01*	1.142	0.78
spring migration - autumn migration	3.654	0.002**	1.431	0.60
breeding - winter	0.326	1.00	1.473	0.58