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RESEARCH ARTICLE

Melatonin attenuates phenotypic flexibility of energy metabolism in a photoresponsive mammal, the Siberian hamster

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ABSTRACT

The duration of melatonin (MEL) secretion conveys information about day length and initiates a cascade of seasonal phenotypic adjustments in photoresponsive mammals. With shortening days, animals cease reproduction, minimize energy expenditure, enhance thermoregulatory capacity and adjust functioning of the hypothalamic-pituitary-adrenal (HPA) axis to match the winter increase in energy demands. Within each season, stress plays an important role in the flexible adjustments of a phenotype to environmental perturbations. Recent studies have shown that thermal reaction norms of energy metabolism were narrower in winter-acclimated Siberian hamsters, *Phodopus sungorus*. We tested the hypothesis that physiological changes occurring in response to prolonged MEL signals, including changes in the secretion of stress hormones, are responsible for the seasonal decrease in phenotypic flexibility of energy metabolism in photoresponsive mammals. To quantify reaction norms for basal metabolic rate (BMR) and cortisol (CORT) secretion, male Siberian hamsters maintained at a long (16 h:8 h light:dark) photoperiod were acclimated repeatedly for 12 days to 10 and 28°C. As predicted, the phenotypic flexibility of BMR decreased when animals were supplemented with MEL. However, at the same time, mean CORT concentration and the reaction norm for its secretion in response to changes in acclimation temperature increased. These results suggest that decreased sensitivity of HPA axis to CORT signal, rather than changes in CORT level itself, is responsible for the decreased phenotypic flexibility in photoresponsive species. Our results suggest that decreased phenotypic flexibility in winter, together with increased stress hormone secretion, make photosensitive species more vulnerable to climate change.

KEY WORDS: Photoperiodism, Phenotypic flexibility, Energy expenditure, Stress, Melatonin, Cortisol

INTRODUCTION

Animals reversibly adjust their physiology, behavior or morphology in response to predictable (e.g. seasonal) or unpredictable (e.g. year-round) and rapid changes in the environment (Wingfield and Kitaysky, 2002; Piersma and Drent, 2003). In the course of seasonal acclimatization to winter, many small mammals cease reproduction (Bronson, 2009), minimize their energy expenditure by reducing body mass (m_b) and basal metabolic rate (BMR), and improve insulation (reviewed in Heldmaier, 1989; Lovegrove, 2005) and facultative heat production (Heldmaier et al., 1981). Seasonal acclimatization is driven by photoperiod and is under endocrine control (Scherbarth and Steinlechner, 2010). Shortening of the light phase of the day results in prolonged melatonin (MEL) secretion at night, whereas increasing the day length does the opposite (Steinlechner et al., 1987). MEL is produced and released from the pineal gland during the dark phase of the day, and the duration of elevated nocturnal MEL levels differs seasonally, serving to translate the environmental signal of day length into an endocrine one (Steinlechner et al., 1987). Changes in photoperiod are a predictable and reliable signal that conveys information about future environmental demands; these changes allow animals to synchronize their physiology with environmental or life-history challenges; e.g. winter survival (Heldmaier and Lynch, 1986), reproduction (Tamarkin et al., 1985) or migration (Coppack and Pulido, 2004). The photoperiod–MEL mechanism controlling seasonal physiology is evolutionarily conserved across both diurnal and nocturnal taxa (reviewed in Bradshaw and Holzapfel, 2007). In photoresponsive mammals, MEL supplementation mimics shortening photoperiod and induces seasonal changes, such as a decrease in the set-point for m_b regulation (Wade and Bartness, 1984), gonadal regression (Hiebert et al., 2006), changes in hypothalamic-pituitary-adrenal (HPA) axis activity (Pyter et al., 2007; Scotti et al., 2008, 2015) and adjustments in thermoregulatory mechanisms (Heldmaier and Lynch, 1986). Thus, MEL plays an important role in an integrative system that controls investments in reproduction and immunity, and in coping with environmental stress (Nelson and Demas, 1997).

Unpredictable, often rapid, environmental perturbations require adequate adjustments of an animal's phenotype year-round (Wingfield and Kitaysky, 2002; Piersma and Drent, 2003). Flexible adjustments of the mechanisms of heat production are a common response of endothermic animals to changing thermal conditions (McKechnie et al., 2007; van de Ven et al., 2013; Boratyński et al., 2016; for review, see Piersma and Van Glis, 2011). However, recent results indicate that phenotypic flexibility changes seasonally, being lower in winter than in summer (Boratyński et al., 2016). Because photoperiod is the primary cue for seasonal phenotypic adjustments in photoresponsive mammals (Lynch, 1973; Heldmaier and Lynch, 1986; Goldman et al., 2000) via actions on the endocrine system, we hypothesized that the decrease of short-term phenotypic flexibility in winter is a result of substantial changes at an endocrine level (Boratyński et al., 2016). Hormones that may be important for the seasonal regulation of phenotypic flexibility are released in response to the environmental stress, which activates two main neuroendocrine pathways. One is the synthesis and release of adrenaline and noradrenaline as part of the sympathoadrenal system; another is the synthesis and the release of glucocorticoids (GCs) as a result

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List of symbols and abbreviations

BMR	basal metabolic rate
CORT	cortisol
GC	glucocorticoid
HPA	hypothalamic-pituitary-adrenal
m_b	body mass
m_t	testes mass
MEL	melatonin
T_a	ambient temperature

of activation of the HPA axis (Haller and Kruk, 2003). GCs directly stimulate coping with an acute stress by mediating recovery from stress, and also prepare an animal for future stress (Sapolsky et al., 2000; Romero et al., 2009). Thus, hormonal stress responses should be understood as a suite of physiological and behavioral mechanisms enabling animals to maintain homeostasis (Romero, 2004; Landys et al., 2006). Endocrine responses to stress may occur when animals respond to unpredictable stimuli (reactive homeostasis; e.g. in response to unpredictable changes in ambient temperature, T_a) or when animals anticipate increases in environmental demands (predictive homeostasis; e.g. during seasonal acclimatization to winter; McEwen and Wingfield, 2003; Romero et al., 2009). For instance, prolonged acclimation to short days, as well as supplementation with MEL, also results in increased cortisol (CORT) levels (Hiebert et al., 2006). Additionally, irrespective of day length, short-term decreases in T_a may induce stress reactions leading to GC levels above the seasonal baseline (Romero et al., 2000; McEwen and Wingfield, 2003).

Based on the findings above, we hypothesized that the winter decrease of phenotypic flexibility of BMR (Boratyński et al., 2017) results from MEL-mediated seasonal changes in the magnitude of stress response. We expected that both BMR and CORT concentrations would increase with short-term acclimation to cold and that both would decrease after acclimation to thermoneutral conditions. We also predicted that MEL supplementation 2 h before lights off, which would lengthen the elevated level of MEL concentration at night, mimicking shortening days, would result in decreased phenotypic flexibility of BMR in animals maintained in a long, summer-like photoperiod. Lastly, we expected that reduced phenotypic flexibility of BMR in MEL-treated animals would be correlated with decreased reaction norms for CORT synthesis. To test the above predictions, we designed an experiment with the Siberian hamster [*Phodopus sungorus* (Pallas 1773)] as a model species in which the same individuals underwent two different treatments, with and without MEL supplementation, which allowed us to estimate the effect of MEL on the reaction norms of BMR and CORT levels. Siberian hamsters are small, photoresponsive rodents that, during seasonal acclimatization, rely on photoperiod (Wiesinger et al., 1989; Scherbarth and Steinlechner, 2010) and develop a distinct winter phenotype characterized by decreased m_b, molt to white pelage, regression of gonads and development of the capacity to enter daily torpor (Heldmaier and Steinlechner, 1981; Jefimow et al., 2004). Previously, we found that the Siberian hamster energy metabolism is highly flexible in response to short-term variations in T_a and that this flexibility differs seasonally both within and among individuals (see above and Boratyński et al., 2016, 2017). These features make the Siberian hamster an ideal model with which to study endocrine control of seasonal changes in the phenotypic flexibility of energy expenditure.

MATERIALS AND METHODS

Experimental design

The study was conducted at the Nicolaus Copernicus University in Toruń, Poland. All experimental procedures were approved by the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland (decision number 43/2015). We used 29 male Siberian hamsters born in our breeding colony in early autumn 2014. Three-week-old animals were separated from breeding pairs and housed individually in standard rodent cages (1246, Tecniplast, Buguggiate, Italy) with access to food (standard rodent diet; Labofeed, Morawski, Kcynia, Poland) and water *ad libitum*. During the entire study, hamsters were kept under a long photoperiod (16 h:8 h light:dark). Experiments began when hamsters were 6 to 9 months old. Animals were supplemented with MEL following Hiebert et al. (2006). Specifically, hamsters were offered a small piece of apple (<5×5×5 mm) 2 h before lights off, which they ate readily. Apple pieces were injected using a Hamilton syringe (1710LT, Hamilton Company, Reno, NV) with crystalline MEL (Sigma Aldrich, Poland, Poznań) dissolved in ethanol to a concentration of 2.7 mg ml^{-1}. Hamsters were supplemented with MEL at a dose of 0.33 mg kg^{-1}. Melatonin solutions were mixed up to 3 days before use and were stored in the dark at 4°C. To control for the effect of MEL administration, hamsters were offered a piece of apple injected only with ethanol (sham treatment). Prepared apple pieces were placed on a labeled tray for ∼30 min to allow ethanol to evaporate at ∼27°C and only then were offered to hamsters. After completing the experiments animals, were killed by cervical dislocation and their testes were dissected and weighed (paired wet testes mass, m_t) to the nearest 0.001 g with an electronic balance (SPU123, Ohaus, Parsippany, NJ, USA).

We used a 2×2 Latin square design in which each individual supplemented with MEL was also measured under sham treatment. Twenty-nine individuals were randomly divided into two groups. Fifteen hamsters were first offered apples injected with MEL and then, after 1 month (no apple in this period), were fed with apple injected only with ethanol (sham treatment). In the remaining 14 hamsters, the order of the treatment was the opposite: first, animals were sham treated and then, after a month, they were supplemented with MEL. Nine out of 29 animals were randomly assigned to a control group and were kept continuously at T_a=20°C; five of them were treated with MEL first, and four hamsters were treated with MEL during second experimental trial (Fig. 1). The remaining animals (N=20) were assigned to experimental subgroups (see below), and after 1 month at T_a=20°C they were exposed to different T_a values to measure flexible changes in BMR (note that photoperiod remained constant). Namely, half of these hamsters (N=10) were acclimated for 12 days to 28°C, and then for 12 days to 10°C; the other half (N=10) underwent a similar acclimation regime, but in the reverse order of temperatures. The sequence of acclimation of individual hamsters to different T_a values was kept the same during MEL and sham treatments.

Data collection

BMR was measured indirectly as a rate of oxygen consumption using an open-flow respirometry system as described in detail in Boratyński et al. (2017). At noon, 1 day after BMR measurements, after each 12 day acclimation to different T_a values, blood samples (∼150 µl) were taken from the retro-orbital sinus of each hamster to measure CORT concentration in the serum. This procedure lasted

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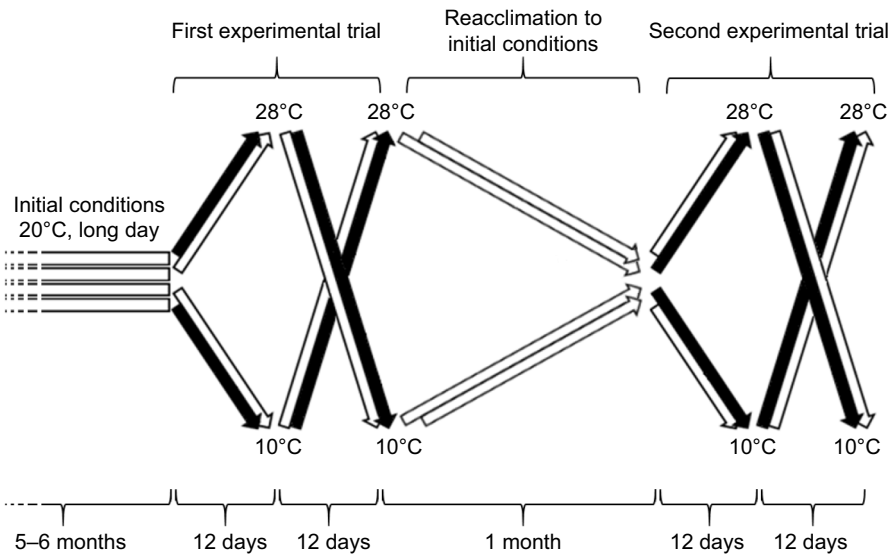

Fig. 1. Acclimation protocol. Arrows indicate separate groups, which were acclimated to 10 or 28°C when hamsters were supplemented with (black arrows) or without (sham treatment, white arrows) melatonin. Note that all hamsters were kept under a constant, long photoperiod (16 h:8 h light:dark). Control groups (not shown) were kept constantly at 20°C, under the same long photoperiod. See Materials and methods for details.

<1 min. If we were unable to draw blood during 1 min, it was done on the following day. This was the case for three samples. Blood samples were taken using a non-heparinized micro-hematocrit tube (catalog no. 7493 21, Brand GmbH, Wertheim, Germany) and transferred to 0.2 ml tubes, and allowed to clot for ∼1 h. Then they were centrifuged for 15 min at 4°C at 2300 ***g***. Serum (50–70 µl) was collected with an automatic pipette (Eppendorf AG, Hamburg, Germany) and placed in tubes, frozen and stored at −20°C for no longer than 2 months before analysis. Prior to analysis, serum samples were diluted 15-fold in Elecsys Diluent Universal [11732277 or 03183971, Roche Diagnostics (RD), Mannheim, Germany]. Serum CORT concentration was measured with an Elecsys Electrochemiluminescence Immunoassay Analyzer (ECLIA; Cobas e411, RD) using an Elecsys Cortisol Assay (11875116, RD).

Data analysis

The effects of MEL administration on the m_b, BMR and serum CORT of control hamsters were analyzed using repeated-measures ANOVA (RM-ANOVA). Data for serum CORT of control hamsters were not normally distributed and thus were log-transformed prior to analysis. Because five of the control hamsters were treated with MEL during the first trial, while the remaining were treated during the second trial, the analyses for each subgroup were performed separately. This allowed us to test for the possible delayed effects of MEL in hamsters that were supplemented during the first trial. Because of the small sample size of control subgroups, we were unable to run analyses using m_b as a covariate. Thus, to test whether observed changes in BMR and CORT resulted from changes in m_b, we calculated them per unit of m_b (BMR/m_b or CORT/m_b). m_b, BMR and BMR/m_b were compared between initial acclimations, and first and second 12-day acclimations of both experimental trials (Fig. 1). Because blood samples were not obtained after initial acclimations, we were able to compare total serum CORT and CORT/m_b only after each of four 12-day acclimations to different T_a values.

The Siberian hamster is a polymorphic species and part of its population does not respond to seasonal changes in day length, i.e. they do not regress their gonads during winter (Goldman et al., 2000). Hence, following Hiebert et al. (2006), individuals in which m_t after MEL treatment was >250 mg were defined as non-responders, while those with m_t<250 mg were defined as responders. m_t was compared between hamsters that were not treated with MEL during the last experimental trial (animals of unknown responsiveness) and those that did or did not respond to MEL (treated with MEL in the second experimental trial). Because a Levene's test indicated a lack of homogeneity of variance (P<0.05), m_t was compared using a non-parametric Kruskal–Wallis test.

The relationship between stress hormone levels and gonadal regression was analyzed using data for CORT obtained after the last acclimation, 1 day before all animals were killed and their testes were dissected. Data for serum CORT and m_t were not normally distributed and thus were log-transformed prior to analysis. As log-transformed serum CORT was negatively related to acclimation T_a (see Results) and m_b, and as m_t positively correlated with m_b, we tested for the relationship between residual log-transformed m_t and residual log-transformed CORT using an ordinary least squares regression. Residuals for log-transformed m_t were obtained from linear regressions between log-transformed m_t and m_b. Residuals for log-transformed CORT were obtained from a general linear model (GLM) in which log-transformed CORT was regressed against m_b and acclimation T_a.

To make inferences about animal phenotypic flexibility, we measured m_b, BMR and blood serum CORT levels of the same individuals after acclimation to 10 or 28°C, and tested for the effect of T_a using GLM procedure (IBM SPSS v. 22). To compare m_b, BMR and CORT of hamsters that were exposed to different acclimation T_a values, and were treated with or without MEL, we used a repeated-measures analysis by including individual ID as a random effect in the GLM. We analyzed the effects of MEL on m_b, BMR and CORT in animals acclimated to different T_a values (10 or 28°C) using GLMs with subsequent experimental trial (1 or 2), consecutive 12-day acclimation (1 or 2) within each trial, acclimation T_a and treatment (MEL or sham) included as fixed factors, and interactions between treatment and acclimation T_a, and between treatment and consecutive 12-day acclimation. In GLMs for m_b-adjusted BMR and m_b-adjusted CORT, m_b was included as a covariate. Assumptions of the linear modeling were checked *post hoc* by inspecting the distribution of residuals obtained from GLMs (check of histograms and quantile–quantile plots; Grafen and Hails, 2002). Data are reported as estimated marginal means and their

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upper and lower 95% confidence intervals (CI). In all analyses, statistical significance was accepted at $P \leq 0.05$. Non-significant trends were accepted at $P \leq 0.1$.

RESULTS

Of the 15 MEL-treated hamsters that were killed after the last acclimation (11 from experimental and four from control groups), eight were classified as responders (m_t<250 mg). Administration of MEL for 24 days resulted in gonadal regression, and testes of hamsters that were not treated with MEL were ∼62% heavier than those of hamsters supplemented with MEL (χ^2=11.11, d.f.=1, P<0.001; Fig. 2). Responding hamsters treated with MEL had 87% smaller testes than sham-treated individuals (P<0.001), and 86% smaller than hamsters non-responding to MEL (P=0.004; Kruskal–Wallis test: χ^2=17.32, d.f.=2, P<0.001; Fig. 2).

Body mass of MEL-supplemented hamsters decreased by ∼8% in controls (Table 1) and by ∼6% in experimental hamsters (MEL: 36.76 g, 95% CI: 36.12–37.40 g versus sham: 39.16 g, 95% CI: 38.51–39.80 g; Table 2). In the experimental group, irrespective of treatment, m_b decreased on average by <1.5 g with subsequent acclimations (12 days: 38.67 g, 95% CI: 38.03–39.31 g versus 24 days: 37.25 g, 95% CI: 36.61–37.89 g; Table 2). However, when treated with MEL, experimental hamsters tended to decrease m_b with subsequent acclimations more than when sham treated (interaction: treatment×subsequent acclimation; Table 2). Hamsters after 12 days of MEL treatment weighed 4% less than when sham treated (MEL: 37.90 g, 95% CI: 36.99–38.81 g; sham: 39.44 g, 95% CI: 38.53–40.34 g), and ∼8% less after 24 days (MEL: 35.62 g, 95% CI: 34.71–36.53 g; sham: 38.88 g, 95% CI: 37.97–39.78 g).

In control hamsters, whole-animal BMR decreased when they were supplemented with MEL (Table 1). However, BMR/m_b of control animals from both subgroups (Table 1) as well as m_b-adjusted BMR of hamsters from the experimental group did not change with MEL treatment (Table 3). m_b-adjusted BMR correlated negatively with acclimation T_a (Table 3, Fig. 3A; for individual reaction norms, see Fig. S1A,C), and on average it changed by ∼13% with an 18°C change in T_a (at T_a=10°C: 304 mW, 95% CI: 300–308 mW, at T_a=28°C: 264 mW, 95% CI: 259–268 mW; Table 3). MEL treatment affected this difference, and m_b-adjusted BMR changed by <12% when hamsters were treated with MEL and by >15% when they were sham treated (interaction: T_a×treatment; Table 3, Fig. 3A; for individual reaction norms, see Fig. S1A,C).

In control animals, log-transformed CORT/m_b increased when they were supplemented with MEL, and was higher after 24 days than after 12 days of supplementation (Table 1). Body mass-adjusted CORT in the experimental group did not change significantly with the duration of MEL supplementation (Table 4). However, m_b-adjusted CORT was 22.5% higher in experimental hamsters when they were fed with MEL (50.17 ng ml^{-1}, 95% CI: 44.17–56.18 ng ml^{-1}) than when they were sham treated (38.90 ng ml^{-1}, 95% CI: 32.90–44.90 ng ml^{-1}; Table 4). When hamsters were supplemented with MEL, their m_b-adjusted CORT changed by >62% in response to an 18°C change in T_a, whereas when they were sham treated, the same change in T_a resulted in a <52% change in m_b-adjusted CORT (interaction: T_a × treatment; Table 4, Fig. 3B, Fig. S1B,D).

In hamsters killed after the last acclimation, log-transformed serum CORT correlated negatively with acclimation T_a ($F_{1,26}$=16.96, P<0.001) and m_b ($F_{1,26}$=6.13, P=0.020). In the same hamsters, log-transformed m_t was positively related to m_b ($F_{1,27}$=25.15, P<0.001). Residuals from the above relationships correlated negatively with each other (r=−0.47, t=2.80, N=29, P=0.009), indicating that hamsters with the smallest gonads had relatively high CORT levels (Fig. 4).

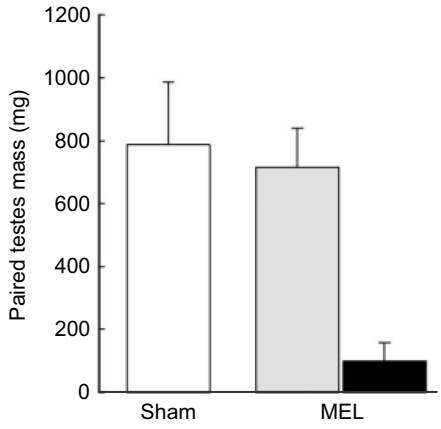

Fig. 2. Paired testes mass in groups of responding (n=8; black bar) and non-responding (n=7; gray bar) Siberian hamsters that were melatonin (MEL) treated (filled bars) and sham treated (n=14; empty bar) for 24 days. For more details, see Results.

DISCUSSION

Environmental stressors, including unfavorable weather, decreased food availability or both, may disrupt animal homeostasis. To minimize the potential detrimental effect of these stressors, several

Table 1. Body mass (m_b), whole-animal basal metabolic rate (BMR), m_b-specific BMR (BMR/m_b), serum cortisol level (CORT) and m_b-specific CORT (CORT/m_b) in Siberian hamsters from control groups (N=9) randomly assigned to two subgroups (A, N=4; B, N=5)

	Initial	12 days	24 days	Control	12 days	24 days	F	P
A								
m_b (g)	37.8±4.9	38.5±3.6	39.0±3.3	38.7±4.3	37.1±5.8	33.3±4.8*	9.76	<0.001
BMR (mW)	269±19	268±15	275±16^a	272±21^b	261±24	241±20^{a,b}	3.96	0.017
BMR/m_b (mW g^{-1})	7.1±3.9	7.0±4.2	7.1±4.9	7.0±4.8	7.0±4.1	7.2±4.3	1.00	0.449
CORT (ng ml^{-1})		29.0±6.2	29.8±8.3		29.2±7.3	54.0±14.5*	7.80	0.007
CORT/m_b (ng ml^{-1} g^{-1})		0.76±0.18	0.79±0.27		0.84±0.34	1.66±0.52*	8.84	<0.005
B								
m_b (g)	44.9±3.3	44.8±4.1	41.3±5.8^a	42.5±5.5	45.1±6.8	47.6±7.9^a	2.56	0.058
BMR (mW)	309±12	303±21	286±24^a	305±15	316±32	331±38^a	4.94	0.004
BMR/m_b (mW g^{-1})	6.9±3.8	6.8±5.0	6.9±4.1	7.2±2.8	7.0±4.7	7.0±4.8	0.85	0.531
CORT (ng ml^{-1})		34.6±5.6	51.1±13.7^{a,b}		17.5±4.0^b	20.5±6.4^b	11.98	<0.001
CORT/m_b (ng ml^{-1} g^{-1})		0.78±0.14^a	1.29±0.45^{a,b,c}		0.39±0.05^b	0.43±0.11^c	13.09	<0.001

Groups were treated with (shaded) or without melatonin, and the sequence of supplementation was opposite (Fig. 1). Different superscript letters indicate significant differences between measurements (RM-ANOVA with *post hoc* Tukey test for pairwise comparisons). Values are means±s.d.

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Table 2. Results of general linear model (GLM) explaining variation in body mass of Siberian hamsters from experimental groups after 12 and 24 days of experiment (subsequent acclimation), when they were acclimated to 10 or 28°C (T_a during acclimation) and when they were treated with or without melatonin (treatment) during the first and second trials (experimental trial)

	d.f.	*F*	*P*
Model intercept	1	697.14	<0.001
T_a during acclimation	1	0.39	0.538
Experimental trial	1	0.70	0.407
Subsequent acclimation	**1**	**9.86**	**0.003**
Treatment	**1**	**27.83**	**<0.001**
Treatment × T_a during acclimation	1	0.16	0.693
Treatment × subsequent acclimation	1	3.62	0.063
Individual ID	**19**	**40.46**	**<0.001**
Error d.f.	54		

Individual ID – test for random effect. Statistically significant effects are indicated in bold.

mechanisms evolved allowing animals to perceive stress and adjust their physiology (i.e. allostasis) (Wingfield and Kitaysky, 2002; Piersma and Drent, 2003; Boonstra, 2005; Landys et al., 2006; Taff and Vitousek, 2016). For example, in the present study, Siberian hamsters increased their stress hormone levels, which correlated with increased basal heat production during acclimation to cold, but showed decreased CORT during thermoneutrality (Fig. 3). Recently, we found that reaction norms for energy metabolism differ seasonally, and that the phenotypic flexibility of BMR and non-shivering thermogenesis had a narrower scope in winter than in summer (Boratyński et al., 2016, 2017). The results of the present experiments using hormonal manipulations strongly support our hypothesis that seasonal changes in endocrine function interact with short-term phenotypic flexibility resulting from thermal acclimation (Boratyński et al., 2016). To the best of our knowledge, this is the first study testing both reaction norms for energy metabolism and stress hormone response to change in acclimation T_a within the same animals. Supplementation of MEL, the primary vertebrate chronobiotic, even under a long day photoperiod, resulted in decreased phenotypic flexibility of m_b-adjusted BMR. Hamsters, when supplemented with MEL, also showed greater changes in m_b-adjusted CORT in response to changes in acclimation T_a than when they were sham treated. These results indicate that the phenotypic flexibility of energetics is affected by day length and strongly

Table 3. Results of GLM explaining variation in basal metabolic rate of Siberian hamsters from experimental groups after 12 and 24 days of experiment (subsequent acclimation), when they were acclimated to 10 or 28°C (T_a during acclimation) and when they were treated with or without melatonin (treatment) during the first and second trials (experimental trial)

	d.f.	*F*	*P*
Model intercept	1	7.58	0.008
T_a during acclimation	**1**	**185.02**	**<0.001**
Experimental trial	1	1.49	0.228
Subsequent acclimation	1	0.76	0.388
Treatment	1	0.60	0.442
Treatment × T_a during acclimation	**1**	**4.43**	**0.040**
Treatment × subsequent acclimation	1	0.64	0.428
Body mass	**1**	**31.44**	**<0.001**
Individual ID	**19**	**4.35**	**<0.001**
Error d.f.	53		

Individual ID – test for random effect. Statistically significant effects are indicated in bold.

suggest that photoperiodic (seasonal) modulation of the HPA axis affects reaction norms for energy metabolism in response to perturbations in the thermal environment.

Short-term MEL supplementation did not result in a change of pelage to winter white, but it resulted in a slight decrease of hamster m_b (Hiebert et al., 2006; present study). Decrease of m_b in short days is related to the lower level of hypothalamic triiodothyronine (Hanon et al., 2000; Murphy et al., 2011; Król et al., 2012), which results from upregulation of the type III deiodinase, which decreases the availability of triiodothyronine (Barrett et al., 2007; Ebling and Barrett, 2008). Because short-photoperiod-induced increases in type III deiodinase are likely mediated by MEL (Barrett et al., 2007), we cannot exclude the possibility that thyroid hormones were involved in the m_b regulation during MEL treatment. The ∼6–8% decrease in m_b observed in hamsters treated with MEL cannot be explained by testicular regression because testes of Siberian hamsters regressed only by <0.8 g, which is <3% of hamster m_b (Wade and Bartness, 1984; Hiebert et al., 2006; present study). Nevertheless, m_b changes resulting from MEL supplementation were small and did not likely affect our results on phenotypic flexibility in BMR.

Despite the slightly decreased m_b and concomitant decrease in whole-animal BMR in hamsters from the control group, their BMR/m_b did not change in response to MEL supplementation. Similarly, m_b-adjusted BMR did not change in response to MEL in experimental groups. The acclimation T_a explained most of the variation in BMR of experimental animals (Table 3). Sham-treated individuals changed their basal heat production by ∼15% in response to an 18°C change in T_a. In other words, BMR changed by 0.8% per 1°C of change in T_a, which is 20% lower than in our previous experiments in summer-acclimated hamsters (∼1% of change in BMR per 1°C of change in T_a; Boratyński et al., 2016). This difference could be expected as in the present study hamsters were acclimated to different T_a values for 12 days compared with 3 weeks in previous studies. This finding indicates that phenotypic adjustment of metabolic rate is a continuous process, but less than 2 weeks is sufficient to induce a detectable change in BMR.

Experimental hamsters altered their energy expenditure in response to change in T_a less when they were supplemented with MEL than when they were sham treated (change in m_b-adjusted BMR by ∼0.6% and ∼0.8% per 1°C of change in T_a, respectively). This supports previous results, showing that flexibility of basal as well as facultative heat production after acclimation to winter had a narrower scope than after acclimation to summer-like conditions (Boratyński et al., 2016). Boratyński et al. (2017) also showed that, compared with summer, the reaction norm for BMR in winter-acclimated hamsters was reduced only in hamsters responding to shortening photoperiod, whereas no seasonal difference was found in non-responding ones. Together with results of this study, this indicates that short photoperiod as well as artificially prolonged high MEL levels both suppress phenotypic flexibility of energy metabolism.

GCs may affect metabolic rate in different ways (Romero, 2004; Landys et al., 2006), and the correlation between resting metabolic rate and GCs indicates that animal energy metabolism is significantly affected by the hormonal regulation of the stress response (Haase et al., 2016). Moreover, both metabolic rate and the stress response are plastic, or more precisely, flexible traits (Wingfield and Kitaysky, 2002; Piersma and Drent, 2003; Taff and Vitousek, 2016), and both are negatively correlated with T_a (Romero et al., 2000; McKechnie et al., 2007). Interestingly, hamsters showed significantly smaller changes in CORT in

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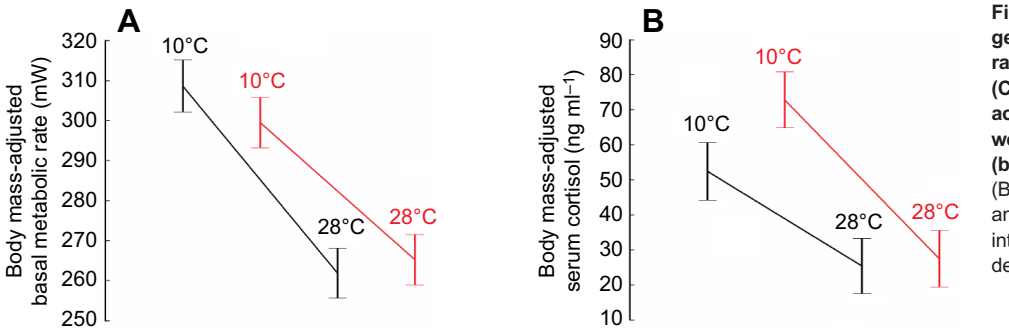

Fig. 3. Estimated marginal means from general linear models for basal metabolic rate (BMR) and serum cortisol level (CORT) in 20 Siberian hamsters acclimated to 10 or to 28°C, when they were treated with (red lines) or without (black lines) melatonin. (A) BMR; (B) CORT. Means are connected with lines, and whiskers indicate 95% confidence intervals. See Materials and methods for details of experimental design.

response to change in acclimation T_a during sham than during MEL treatment (Fig. 3B, Fig. S1B,D), which is inconsistent with an earlier report on stress hormone levels in deer mice *Peromyscus maniculatus* acclimated to cold under long and short days (Demas and Nelson, 1996). In that study, cold acclimation led to an increase of the corticosterone concentration only under long days (Demas and Nelson, 1996). It was found that functioning of the reproductive system as well as secretion of sex hormones (e.g. testosterone) were inhibited in response to high levels of GCs, be it winter or stress related (reviewed in Crespi et al., 2013). High testosterone levels can decrease corticosteroid-binding globulins and thus can have a suppressive effect on stress-induced free GCs in many mammalian species (Kitay, 1963; Gala and Westphal, 1965; McDonald et al., 1981; Mataradze et al., 1992; Place and Kenagy, 2000; but see Boonstra, 2005). Thus, differences in stress responses to cold between mice (Demas and Nelson, 1996) and hamsters (present study) may result from differences in the degree of testicular regression and thus different interactions between testosterone and corticosteroid-binding globulins. Deer mice regressed their testes by only ∼30% (Demas and Nelson, 1996), whereas photoresponding Siberian hamsters regressed their testes by >70% (Fig. 2) (Jasnow et al., 2000; Hiebert et al., 2006; Scotti et al., 2008; present study). Furthermore, hamsters with the lowest m_t had the highest CORT level (Fig. 4), which was independent of acclimation T_a or m_b. Thus, species-specific interactions between reproductive and stress endocrine axes may offer an explanation for the differences in stress response to T_a. However, it should be pointed out that CORT and corticosterone concentrations may respond differently to stress, and this may differ between species. For instance, in tuco-tucos (*Ctenomys talarum*), only CORT changed in response to acute stress, whereas corticosterone remained unchanged despite stress (Vera et al., 2011). This might further indicate that observed different responses to cold among rodents could be explained by differences in hormonal regulation of the stress response. In Siberian hamsters, unlike in deer mice, CORT is the dominant glucocorticoid (Demas and Nelson, 1996; Reburn and Wynne-Edwards, 2000), which suggests that potential differences in the interaction between stress hormones and the reproductive axis may be responsible for observed differences between these rodent species.

The flexible changes in BMR in hamsters were suppressed by MEL supplementation (Fig. 3A, Fig. S1A,C), which correlated with the highest level of stress hormones and with the greatest reaction norms for CORT synthesis in response to thermal acclimation. This was partially inconsistent with our initial prediction (Boratyński et al., 2016) that changes in stress hormone levels should be smaller in winter-acclimated Siberian hamsters. However, these findings could be explained by the actions of GC-mediated negative feedback on the HPA axis. In photoresponding mammals exposed to winter-like conditions, including the Siberian hamster, the negative feedback of the HPA axis is upregulated and tuned for seasonally increased GCs (Pyter et al., 2007). At lower concentrations of stress hormones, GCs bind to mineralocorticoid receptors, whereas when GCs are present in high concentrations

Table 4. Results of GLM explaining variation in serum cortisol of Siberian hamsters from experimental groups after 12 and 24 days of experiment (subsequent acclimation), when they were acclimated to 10 or 28°C (T_a during acclimation) and when they were treated with or without melatonin (treatment) during the first and second trials (experimental trial)

	d.f.	*F*	*P*
Model intercept	1	11.20	0.002
T_a during acclimation	**1**	**92.15**	**<0.001**
Experimental trial	1	1.09	0.300
Subsequent acclimation	1	1.39	0.243
Treatment	**1**	**5.87**	**0.019**
Treatment × T_a during acclimation	**1**	**5.94**	**0.018**
Treatment × Subsequent acclimation	1	0.40	0.532
Body mass	**1**	**5.94**	**0.018**
Individual ID	**19**	**2.13**	**0.016**
Error d.f.	53		

Individual ID – test for random effect. Statistically significant effects are indicated in bold.

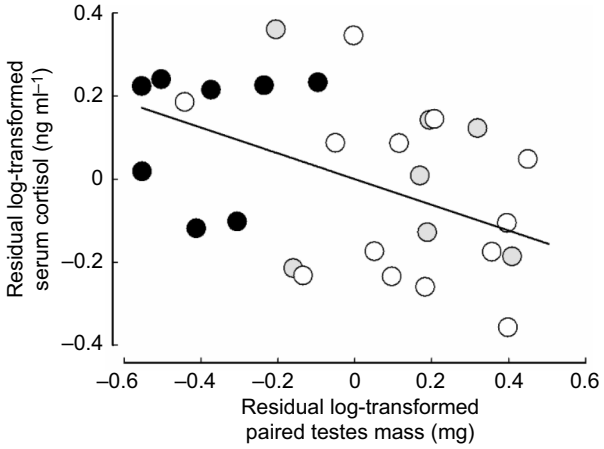

Fig. 4. Relationship between residual log-transformed paired testes mass and residual log-transformed serum cortisol level in groups of responding (*n*=8; black symbols) and non-responding (*n*=7; gray symbols) Siberian hamsters that were melatonin treated (filled symbols) and sham treated (*n*=14; empty symbols) for 24 days. Residuals for cortisol were obtained from the relationship between log-transformed serum cortisol level and acclimation temperature during the last experimental trial. Residuals for testes mass were obtained from the relationship between log-transformed paired testes mass and body mass.

they bind to GC receptors, which have lesser affinity to GCs (Boonstra 2005; Landys et al., 2006). Binding of GCs to the hypothalamic receptors results in negative feedback inhibition of GCs, which is an evolutionarily conserved mechanism protecting animals from negative effects of prolonged exposure to stress hormones (Romero, 2004). Eventually, negative feedback in hamsters should result in decreased levels of GCs after days of MEL supplementation. However, in hamsters from the control group, high basal CORT levels were maintained for more than a week when they were supplemented with MEL. Increased CORT levels in winter, or during MEL supplementation, may suggest that prolonged exposure to short days results in chronic stress in Siberian hamsters (Pawlak et al., 2009). Thus, on the one hand, increased CORT levels for a prolonged time could suggest a disruption of the negative feedback of the HPA axis (Romero, 2004). On the other hand, it is possible that the threshold for the negative feedback of the HPA axis was shifted to the higher CORT level (McEwen and Wingfield, 2003; Romero et al., 2009). It is also possible that the number of GC receptors decreased to compensate for the high stress levels (Sapolsky, 2001), which would result in resistance to GC feedback and increased basal concentration of GCs (Sapolsky and Plotsky, 1990). If so, then greater changes in CORT levels in response to cold acclimation would be required to achieve similar changes in energy metabolism in winter-acclimated or MEL-supplemented animals.

In the Siberian hamster, and likely many other endotherms, basal metabolism and stress hormone concentrations change in a correlative manner with short-term changes of acclimation T_a. Relatively short-term changes in the duration of the MEL signal used in this study only triggered the seasonal changes, and did not result in a full change to the winter phenotype (Hiebert et al., 2006; present study), as was observed after long acclimation to short days (Boratyński et al., 2016). Thus, it seems that a decrease in short-term phenotypic flexibility of energy metabolism in MEL-treated hamsters, and likely in animals that undergo seasonal acclimatization to winter conditions (Boratyński et al., 2016, 2017), could be explained by changes at the endocrine level. Specifically, seasonal shifts in the HPA axis feedback (including changes in GC receptors) correlating with a high level of GCs suggest that animals acclimated to short days require higher hormonal responses to environmental stressors to achieve changes on energetic level similar to that observed in long-day acclimated animals. This would support our hypothesis that seasonal changes in the HPA axis in photoresponsive animals interact with stress responses necessary for phenotypic flexibility of energetics in response to short-term thermal perturbations (Boratyński et al., 2016, 2017; present study). Phenotypic flexibility is considered an adaptation that allows animals to cope with increased climate unpredictability (Canale and Henry, 2010). Thus, studies of animal responses to changing environmental demands should consider photoperiodism as a trait that may significantly affect animal vulnerability and survival in the face of climate change. As suggested by Canale and Henry (2010), whether an animal is photoresponsive may be an important factor that shapes the future state of natural populations of different species.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.S.B., M.J., M.S.W.; Methodology: J.S.B., M.J., M.S.W.; Validation: J.S.B., M.J., M.S.W.; Formal analysis: J.S.B.; Investigation: J.S.B., M.J., M.S.W.; Resources: J.S.B., M.J., M.S.W.; Data curation: J.S.B.; Writing - original draft: J.S.B.; Writing - review & editing: J.S.B., M.J., M.S.W.; Visualization: J.S.B.; Supervision: J.S.B., M.J., M.S.W.; Project administration: J.S.B.; Funding acquisition: J.S.B., M.S.W.

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