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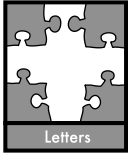
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## Female great tits *Parus major* do not increase their daily energy expenditure when incubating enlarged clutches

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Several studies have shown that enlargement of clutches during incubation reduces the long-term survival of parents. In line with these findings, studies on the energetic costs of nocturnal incubation show an increase in energy expenditure with clutch enlargement. Studies on daily energy expenditure during incubation ( $DEE_{inc}$ ), however, do not consistently show such a negative effect of clutch enlargement. To determine whether differential survival results from a direct increase in energy costs or rather from costs associated with compensatory behaviour, we studied the  $DEE_{inc}$  ( $\text{kJ day}^{-1}$ ), change in body mass and nest attendance behaviour of free-living female great tits *Parus major* that incubated either control or experimentally enlarged clutches.  $DEE_{inc}$  did not differ between the two treatment groups, but was negatively related to mean ambient temperature over the 24-h measuring period, and to the fraction of daytime females spent on the nest. Controlling for these two factors, females incubating enlarged clutches did not spend more energy per 24 h period than females incubating control clutches. Clutch enlargement also did not affect body mass of incubating females, or their nest attendance behaviour. Yet, in the enlarged group body mass change and nest attentiveness were negatively correlated, suggesting that females responded differently to the experimental treatment and thereby preventing us from finding an effect of clutch enlargement.

Life-history theory predicts that parents should produce the number of offspring that maximises their fitness (Roff 1992, Stearns 1992). In birds, parental decisions regarding clutch size may act during three distinct phases: the egg laying, incubation, or nestling stage. In altricial birds, selection on clutch size has long been thought to take place primarily during the nestling phase (Lindén and Møller 1989, Dijkstra et al. 1990, Vanderwerf 1992, Williams 1996). Recently, however, studies focussing on the cost of incubation revealed that females incubating experimentally enlarged clutches bear fitness consequences in terms of reduced survival (Visser and Lessells 2001, de Heij et al. 2006), or reduced fecundity (Hanssen et al. 2003) in the subsequent breeding season.

How clutch size during incubation affects survival is not yet well-understood. A fruitful first step to unravel the mechanisms that underlie clutch size effects on survival is to study how clutch size affects energy expenditure. Energy expenditure is often used as a proxy for fitness, since it is a universal currency that makes aspects of parental activity, offspring needs and resource availability comparable (Daan et al. 1996, Ricklefs 1996). If energy is indeed a useful approximation of fitness, then energy expenditure should increase with clutch enlargement.

In the literature, two groups of studies reported on the relationship between energy expenditure and clutch size during incubation. Studies that used respirometry (Biebach 1981, Biebach 1984, Haftorn and Reinertsen 1985, Weathers 1985, de Heij et al. 2007) to determine the energetic costs of keeping eggs at constant temperature (i.e. contact incubation) provide a consistent pattern. They all show that females expend more energy when incubating larger clutches. Yet, studies that used the doubly labelled water (DLW) technique (Lifson and McClintock 1966) to determine the energy expenditure of the attending parent over a 24-h period ( $DEE_{inc}$ ) show a less consistent pattern. Two of them (Moreno and Carlson 1989, Engstrand et al. 2002) found no effect, while two others (Moreno et al. 1991, Moreno and Sanz 1994) show an increase in  $DEE_{inc}$  with clutch enlargement (for details about the studies see Table 1). Thus while the results of the studies using respirometry would support the idea that an increase in energy expenditure with clutch enlargement may result in negative effects on long-term survival, the studies using the DLW technique do not. Yet, data on nocturnal incubation,  $DEE_{inc}$  and survival are often not collected on one and the same species, and therefore the inconsistency in results may also be caused by inter-species variations.

Table 1. Experiments on the effect of clutch size on  $DEE_{inc}$ , nest attentiveness and body mass.

Species	Manipulation categories <sup>a</sup>			Clutch size <sup>b</sup>		Response variables <sup>c</sup>			References
	Reduced	Control	Enlarged	Original	Manipulation	$DEE_{inc}$	Nest attentiveness	Body mass	
<i>Cinclus cinclus</i> White-throated dipper	6	17	8	5	1	no	no	no	Engstrand et al. 2002
<i>Ficedula albicollis</i> Collared flycatcher	4	3	5	6	2	yes	no	no	Moreno et al. 1991
<i>Ficedula hypoleuca</i> Pied flycatcher	2	2	2	5–7	2	no	no <sup>d</sup>	yes <sup>d</sup>	Moreno and Carlson 1989
<i>Ficedula hypoleuca</i> Pied flycatcher	7	3	8	5–6	1	yes	–	no	Moreno and Sanz 1994
<i>Parus major</i> Great tit	–	14	15	5–12	3	no	no	no	This study

<sup>a</sup>Indicated is the sample size per manipulation category.

<sup>b</sup>In the column ‘clutch size’ is presented the ‘original clutch size’ of females used in the experiment, and the number of eggs that were added/removed from the clutch (‘manipulation’).

<sup>c</sup>The effect of clutch size (yes or no) is given for the three response variables.

<sup>d</sup>The effect of clutch size manipulation on nest attentiveness and body mass is measured for a larger sample size than used for the effect of clutch size on  $DEE_{inc}$ , respectively 8, 8, 10 (reduced, control, enlarged) and 11,13,11.

In the present study, we performed an experiment in which we manipulated clutch size (either enlarged or control) and measured  $DEE_{inc}$  (24 h) of free-living female great tits with the DLW-technique. We focussed on females, as in great tits females are the attending sex; males at most assist by feeding their partner. To exclude the possibility that results are due to differences between species, we performed our study in a great tit population of which previous studies showed that enlargement of clutch size during the incubation period resulted both in elevated energetic costs of nocturnal incubation (de Heij et al. 2007), and in reduced survival for parents (de Heij et al. 2006). We also studied effects of clutch enlargement on body mass and nest attentiveness of the females during the measuring period.

## Materials and methods

### Study population

This study was conducted in the Lauwersmeer, in the north of the Netherlands (53° 20'N, 6° 12'E) during the breeding season of 2004. We used 8 woodlots that consisted of mixed deciduous tree species and together contained about 200 nest-boxes: for further details see Tinbergen (2005). In this study population, clutches contain on average  $9.3 \pm$  (SD) 1.8 eggs ( $n = 1140$ ; 1994–2003).

### General procedure

From the beginning of April, all nest-boxes were checked at least once a week to determine laying date and clutch size. Laying date was estimated by counting back from the observed clutch size, assuming that one egg was laid per day. No clutches were complete at first discovery. The onset of incubation was determined by daily nests visits from the fifth egg onwards. Onset of incubation was defined as the first day the female was found incubating or the first day the eggs were found uncovered and warm.

### Clutch size manipulation

From the available nests, 35 nests were randomly assigned to either of two treatments. Clutches were either kept as control ( $n = 19$ ), or experimentally enlarged ( $n = 16$ ). The clutches in the control group remained unmanipulated, while clutches in the experimental group received three extra eggs from donor nests which had a similar incubation stage. With the manipulation size of three eggs, we adhere to previous clutch size or brood size manipulations in great tits (Sanz and Tinbergen 1999, Wiersma and Tinbergen 2003, de Heij et al. 2007) to facilitate comparison. Clutches were manipulated on average  $3 \pm 1.5$  d before the measurement of energy expenditure, and the original clutches were restored the day thereafter. To prevent nest desertion (de Heij et al. 2006), we did not experimentally reduce clutch sizes.

### Response variables

#### Measurements of $DEE_{inc}$

$DEE_{inc}$  was measured using the DLW-technique (Lifson and McClintock 1966). On day 9 or 10 of incubation, i.e. three-quarters through the incubation period, females were caught with a hand-net on leaving the nest-box during the day (mean time 13:51 h; range 9:01–16:13 h). After capturing, the females were identified, or ringed. Subsequently, they were injected intraperitoneally with 0.10 ml of a mixture containing 5.494 g of 94.20 atom %  $H_2^{18}O$  and 3.361 g of 99.90 atom %  $D_2O$  using 0.3 ml insulin syringes (the exact dose per animal was measured by weighing – accurate to 0.0001 g – syringes before and after injection using a Analytic Balance Mettler Toledo AG245 (Mettler Toledo B.V., Tiel, The Netherlands).  $DEE_{inc}$  was measured following one of two protocols, which we assume to yield similar results. To minimize disturbance, most females ( $n = 30$ ) were measured with the so-called single-sample protocol (Williams and Dwinell 1990). These birds were released immediately after injection, and recaptured after 25 h to take a blood sample from the brachial vein in the wing. For a small number of females ( $n = 5$ ),  $DEE_{inc}$  was measured following the two-sample protocol to

determine equilibrium values of both isotopes. These birds were kept in a cloth bag for an hour after injection to allow for equilibration of DLW with the bird's body water pool (Speakman et al. 2001). Then, an initial blood sample from the brachial vein was taken and birds were released. Twenty-four hours after release, birds were recaptured and a second blood sample was taken from the brachial vein in the other wing. Blood samples of all individuals were collected in triplets in heparinised glass capillaries (20  $\mu$ l; in total 60  $\mu$ l) that were flame-sealed immediately. Samples were stored at room temperature and in the dark.

Blood samples were analysed using isotope ratio mass spectrometry at the Centre for Isotope Research of the University of Groningen, the Netherlands. Background isotopes levels of deuterium ( $D_2$ , 152.67 ppm) and oxygen-18 ( $^{18}O$ , 1999.61 ppm) were determined from blood samples from 4 randomly selected, non-experimental females at the same stage of incubation. Daily  $CO_2$  production was calculated using equation 35 of Lifson and McClintock (1966). The body water pool was assumed to be on average 66% of the total body mass (Mertens 1987). This value was comparable to the mean total body water (65.5%) for the individuals that were measured with the two-sample protocol.  $DEE_{inc}$  ( $kJ day^{-1}$ ) was calculated from the  $CO_2$  production using a respiratory quotient of 0.75 (following Tinbergen and Dietz 1994), and an energetic equivalent of 19.9  $kJ l^{-1}$  of oxygen consumed.

#### Other response variables

To determine the change in body mass over the measuring period, birds were weighed both before and after the DLW-measurement.

To measure nest attentiveness, we mounted a sensor (HOBO logger, Mulder-Hardenberg B.V., The Netherlands) between the eggs that registered nest temperature every 15 s. From changes in nest temperature the nest attentiveness of females could be inferred: a sudden drop in temperature represented the departure of the female from the nest, while a sharp increase in temperature the return of the female to the nest. The following times were recorded: *latency to return* (s) defined as the time between release of the female after injection and first return of the female to the nest, and *length of the night* (s) defined as the time span between the last entry in the evening and the first exit in the morning. During the day, the following behaviours were scored: *time away from the nest* (s) and *time on the nest* (s) defined as the total time during the day that the female spent away from the nest (excluding the latency to return) and on the nest respectively, *the number of recesses* (n)

defined as the number of times the female went away from and returned to the nest during the day. In the analyses, latency to return and time on the nest were expressed as fraction (time divided by the length of the day).

To validate data on nest attentiveness derived from the temperature loggers, the nest attentiveness of several females (n=8) was also recorded by video cameras that were positioned outside the nest-box (mean total observation time 2h 22 min). The two measures of nest attentiveness were highly correlated ( $R^2 = 0.98$ ).

#### Additional measurements

##### Ambient temperature

At a central location in the study area, a temperature sensor (Tinytalk II, Gemini Data Loggers, INTAB Benelux) that was mounted at a height comparable to that of the nest-boxes (ca. 2 m above the ground) recorded ambient temperatures ( $^{\circ}C$ ) at a 10 min interval. For each individual, mean ambient temperature was calculated for the entire 24-h period of measurement, as well as for the night and the day separately.

#### Statistical analysis

Of the 35 birds that were initially injected with DLW, 6 were excluded: 3 birds abandoned their nests, 2 birds could not be recaptured in time and for 1 bird, the  $CO_2$  production could not be determined accurately. Therefore, the actual sample size was 29 (control, n=14; enlarged, n=15). For 4 (3 enlarged, and 1 control) of the 29 individuals, nest attentiveness could not be determined due to malfunctioning of temperature loggers, which reduced the sample size to 25 individuals in analyses of nest attentiveness.

All analyses were performed using GLM's in SPSS 12 for Windows. Effects of clutch size manipulations were studied on  $DEE_{inc}$  ( $kJ day^{-1}$ ), change in body mass (g) and nest attentiveness (fraction latency to return (-), the length of the night (s), fraction of time spent on the nest (-), and the number of recesses during the active day (n)). A correlate test showed that  $DEE_{inc}$  and the fraction of time spent on the nest were negatively correlated (Pearson correlation 0.47,  $P = 0.02$ ). An explanatory analysis revealed that of ambient temperature ( $^{\circ}C$ ; either over a 24-h period, or separated for daytime and night time), original clutch size (n), nest thickness (mm) and body mass (g), only ambient temperature explained part of the variation in the response variables. In all analyses, the experimental treatment (control or enlarged clutch size) was treated as a fixed

Table 2. Mean values and test results for the two treatment group at the start of and during the experimental treatment. Values significant at the 0.05 level are presented in bold.

	Experimental groups		$F_{1,27}$	P
	Control	Enlarged		
Laying date (April date)	17.4 $\pm$ 6.9	17.3 $\pm$ 6.4	0.0	0.97
Body mass at injection (g)	20.1 $\pm$ 1.0	19.7 $\pm$ 1.1	1.3	0.26
Original clutch size (n)	9.1 $\pm$ 1.2	9.0 $\pm$ 2.0	0.1	0.82
Manipulated clutch size (n)	9.1 $\pm$ 1.2	12.0 $\pm$ 2.0	22.3	<b>&lt;0.001</b>
Ambient temperature ( $^{\circ}C$ )	13.3 $\pm$ 1.6	12.7 $\pm$ 1.5	1.1	0.31

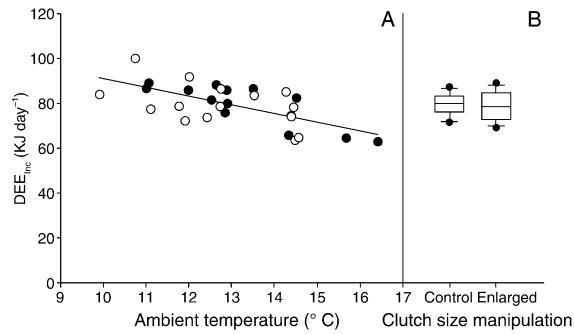


Fig. 1. A) Daily energy expenditure during incubation ( $DEE_{inc}$   $\text{kJ day}^{-1}$ ) of female great tits in relation to mean ambient temperature measured over a 24-h period for control (closed symbols) and experimentally enlarged (open symbols) clutches. B) Residual  $DEE_{inc}$  on ambient temperature and nest attentiveness for females incubating either enlarged or control clutches. To facilitate interpretation residual values are added to the mean  $DEE_{inc}$  of females in the control category. Box plots represent the central 50% of the data, and whiskers indicate the range, apart from the outliers that are marked as dots. Horizontal lines within the box mark median values (straight line).

factor, and mean ambient temperature was included as covariate. Interactions were tested, but not reported unless significant. All values are presented as means  $\pm$  SD, unless stated otherwise.

## Results

### Daily energy expenditure

There was no initial difference between females of the two treatment groups at the start of the experimental treatment, nor did the females experience different ambient temperatures during the experiment (Table 2). Females incubating enlarged clutches spent  $79.3 \pm 9.1$  ( $DEE_{inc}$ ,  $\text{kJ day}^{-1}$ ), while those incubating control clutches spent  $79.6 \pm 9.6$   $\text{kJ day}^{-1}$ . Thus,  $DEE_{inc}$  did not differ between the treatment groups (ANOVA,  $F_{1,27} = 0.004$ ,  $P = 0.95$ ).  $DEE_{inc}$  was negatively correlated to ambient temperature, and the fraction of time females spent on the nest (Fig. 1A, Table 3). When we controlled for both these factors, females incubating enlarged clutches did not expend more energy than females incubating control clutches (residual of the regression of  $DEE_{inc}$ ; control  $0.6 \pm 4.8$ , enlarged  $-0.6 \pm 6.5$ ; Fig. 1B, Table 3). The variance in residual  $DEE_{inc}$  between the two treatment

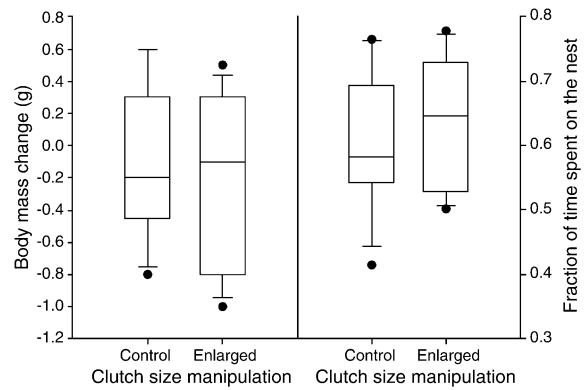


Fig. 2. Change in body mass over the measuring period (A), and fraction of time during daytime that females spent on the nest (B), in relation to the clutch size manipulation. For an explanation of the composition of box plots see legend of Fig. 1.

groups did not differ significantly (Levene's test  $F_{1,23} = 3.4$ ,  $P = 0.08$ ).

### Change in body mass

During the measurement period, birds lost  $0.17 \pm 0.48$  gram between the time of injection ( $19.89 \pm 1.04$  g) and the time of recapture ( $19.72 \pm 1.03$ ). This mass loss was not statistically different from zero (one-sample  $t$  test:  $t_{1,28} = -1.92$ ,  $P = 0.07$ ). Mass change did not differ between females of the two experimental groups, nor was it related to ambient temperature (Fig. 2, Table 3).

### Nest attentiveness

After injection, females returned after  $2 \text{ h } 29 \text{ min} \pm 1 \text{ h } 51 \text{ min}$ . The remainder of the day, females alternated  $23.1 \pm 5.5$  periods on the nest ( $24.9 \pm 8.9$  min) with periods away from the nest ( $8.6 \pm 2.4$  min). In total, females spent  $8 \text{ h } 55 \text{ min} \pm 1 \text{ h } 33 \text{ min}$  on the nest and  $3 \text{ h } 12 \text{ min} \pm 57 \text{ min}$  away from the nest during the active day. None of these behaviours was affected by the experimental treatment or by the mean ambient temperature during daytime (Fig. 3, Table 4). The time females spent in the nest-box at night ( $10 \text{ h } 20 \text{ min} \pm 33 \text{ min}$ ) was not affected by clutch size manipulation, but was positively correlated with mean ambient temperature during the night (Table 4): females spent more time in the nest-box on warmer nights.

Table 3. Test results for the effect of clutch size manipulations and ambient temperature on  $DEE_{inc}$  and change in body mass. Values significant at the 0.05 level are presented in bold.

	$DEE_{inc}$ ( $\text{kJ day}^{-1}$ )			BM change (g)		
	$B \pm \text{SEM}$	$F_{1,25}$	$P$	$B \pm \text{SEM}$	$F_{1,25}$	$P$
Intercept	$150.0 \pm 12.1$	157.8	<b>&lt; 0.001</b>	$0.5 \pm 0.8$	0.58	0.45
Mean ambient temperature	$-3.8 \pm 0.8$	23.6	<b>&lt; 0.001</b>	$-0.06 \pm 0.06$	0.97	0.34
Fraction of time on nest	$-34.3 \pm 12.1$	8.1	<b>0.01</b>	—	—	—
Clutch size manipulation	$-1.4 \pm 2.4^a$	0.3	0.59	$-0.1 \pm 0.2^a$	0.28	0.60

<sup>a</sup>Parameter estimates of the enlarged group relative to the control.

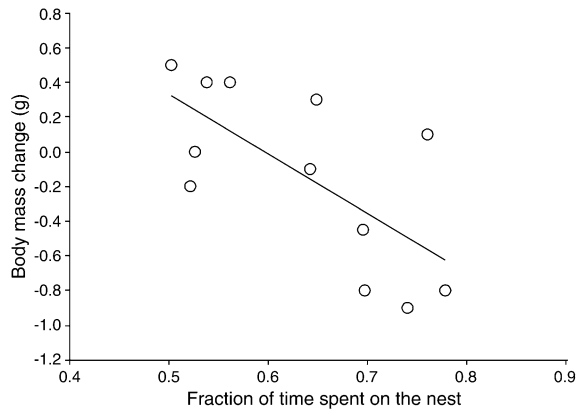


Fig. 3. Correlation between body mass change and the fraction of time during daytime that a female spent on the nest for birds in the experimentally enlarged category.

This association could not be explained by change in day length due to date ( $t_{1,24} = 0.30$ ,  $P = 0.77$ ).

## Discussion

Females of the two treatment groups did not differ in their energy expenditure over a 24-h period, suggesting that females incubating experimentally enlarged clutches did not respond to the manipulation by increasing their  $DEE_{inc}$ . These results are surprising given the results of our previous studies in the same population in which we found both an increase in nocturnal energetic energy expenditure (de Heij et al. 2007), and a lower survival probability to the subsequent breeding season (de Heij et al. 2006) in relation to clutch enlargement.

A possible explanation is that the sample size of our study was too small to detect an effect of clutch size on  $DEE_{inc}$ . A power analysis showed that there was enough power (90%) to detect a difference of 10.7 kJ per day between group means in  $DEE_{inc}$ . This value is equal to 13% of the mean  $DEE_{inc}$  of the control group; previous studies report differences between 9 and 18% (Moreno et al. 1991, Moreno and Sanz 1994). Within our dataset differences in  $DEE_{inc}$  of a magnitude of 37.1 exist, which suggest that we could have detected an effect of clutch enlargement on  $DEE_{inc}$  if females responded by increasing their  $DEE_{inc}$ . We, therefore, believe that this explanation is not the most likely one.

An alternative explanation is that females changed behaviourally or physiologically in response to the clutch enlargement. Reid et al. (2000) show in their study that larger clutches cool more slowly than smaller ones when left unattended, which may possibly allow the attending parent to change her nest attentiveness and forage for a longer period to meet the extra energy demand of incubating enlarged clutches without increasing her  $DEE_{inc}$  (Reid et al. 2002). Females of the two treatment groups, however, did not differ in their nest attentiveness. They also did not differ in their body mass change, suggesting that the females incubating enlarged clutches did not utilise their energy reserves to cover their expenditure. Given that the incubation period is much longer than the measure period, this measure may not be conclusive. Thus, females in the enlarged group did not seem to change behaviourally or physiologically.

Yet, when looking more thoroughly at the females behaviour within the enlarged group, we found that the change in female's body mass was negatively related to nest attentiveness ( $F_{1,10} = 8.8$ ,  $P = 0.01$ , adjusted  $R^2 = 0.42$ , Fig. 3). This correlation indicates that females reacted differently to the experimental treatment. They either spent more time on foraging and gained weight or spent more time on the nest and lost weight, suggesting that females either choose to invest in self-maintenance or in their offspring. Since  $DEE_{inc}$  and nest attentiveness were negatively correlated, the difference in behaviour of females in the enlarged group has likely prevented us from showing any effect of clutch enlargement on  $DEE_{inc}$ .

Furthermore, the contribution of the male to the energy intake of the female may be more important than assumed in this study. In response to clutch enlargement, males might have changed their behaviour and brought more food to their partner (Lyon and Montgomerie 1985), thereby reducing the energy expenditure of their partner at the expense of their own expenditure. This scenario would be in line with our previous finding that the survival of males was negatively affected by clutch enlargement (de Heij et al. 2006). Unfortunately, the feeding behaviour of males is hard to quantify in our study population. Males not only show large variation in their contribution to their partner, but they also often call their partners out of the nest-box either to feed them or to guide them to food (Royama 1966). Nevertheless, including the role of the male during the incubation period may be essential to understand the energy budget of the female.

In conclusion, the results of this study indicate that the relationship between nocturnal incubation and  $DEE_{inc}$  is

Table 4. Test results for the effect of clutch size manipulations and ambient temperature on several aspects of nest attentiveness. Values significant at the 0.05 level are presented in bold.

	Fraction latency to return			Fraction time on nest			Number of recesses			Length of night (s)		
	B $\pm$ SEM	$F_{1,22}$	P	B $\pm$ SEM	$F_{1,22}$	P	B $\pm$ SEM	$F_{1,22}$	P	B $\pm$ SEM	$F_{1,23}$	P
Intercept	0.3 $\pm$ 0.2	1.6	0.21	0.5 $\pm$ 0.2	11.3	<b>0.003</b>	14.1 $\pm$ 8.6	2.7	0.12	32,173 $\pm$ 2,137	220	<b>&lt; 0.001</b>
Mean day temperature	-0.01 $\pm$ 0.01	0.2	0.65	0.01 $\pm$ 0.01	0.3	0.56	0.6 $\pm$ 0.6	0.5	0.47	432 $\pm$ 204 <sup>a</sup>	4.5	<b>0.05</b>
Clutch size manipulation	0.0 $\pm$ 0.05 <sup>b</sup>	0.0	1.00	0.04 $\pm$ 0.04 <sup>b</sup>	0.7	0.40	-0.6 $\pm$ 2.2 <sup>b</sup>	0.1	0.83	-844 $\pm$ 759 <sup>b</sup>	1.2	0.28

<sup>a</sup>Instead of mean day temperature, mean night temperature was used in this analysis.

<sup>b</sup>Parameter estimates of the enlarged group relative to the control.

not straightforward. Since females can compensate behaviourally or physiologically in order to keep similar levels of  $DEE_{inc}$  and they can do so using different mechanisms, revealing an effect of clutch enlargement can be difficult.

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