

Repeatability of Daily Energy Expenditure in Kittiwakes *Rissa tridactyla*



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BIO-3950 Master`s thesis in Biology

Department of Arctic and Marine Biology

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Abstract

Estimates of repeatability (R) are an important statistic tool in assessing the consistency of individual differences and thus different phenotypes. A high repeatability of estimates of the daily energy expenditure (DEE), allow for better inferences of the long term energetic effort of individuals, as well as for sound correlations of other traits related to individual performance, such as reproductive success. Previously, measurements of DEE have been restricted to 24h, but the ecological relevance of such a time frame has been questioned. Estimates of DEE, using the doubly labelled water (DLW) method, were repeated on individuals of kittiwakes breeding on Svalbard, during four study years. The aim of the study was to investigate the time scale in which kittiwakes balance their energy expenditure. Repeatability of DEE was estimated using two different measurement intervals, measuring over a period of either 24h or 72h, within years as well as between years. In addition, it was investigated whether the less invasive single-sample (SS) DLW protocol could improve repeatability over the more common two-sample (TS) protocol, by allowing for kittiwakes to exhibit a near-natural behaviour. We found that individual DEE was repeatable both within (**72h**: $R = 0.772$) and between years (**72h**: $R = 0.430$) using the TS protocol and the long measurement interval. The short interval estimates were not significantly different from zero, although applying the SS protocol appeared to have an increasing effect on R . These findings demonstrate, that prolonging the length of the DEE measurement interval, caused a high increase in R , suggesting that kittiwakes budget their energy expenditure over a longer time period than 24 hours. The moderate to high R found between years suggest that kittiwakes are consistently different in their energy expenditure over longer periods of time. However, when adjusting for body mass (BM) only within-season repeatability was significant (**72h**: $R = 0.652$), which was mainly due to a high inter-individual variation in BM.

Key-words: doubly labelled water, seabirds, ecological physiology, metabolic rate, intraclass correlation coefficient, consistent individuals differences, repeated measurements, long-term field physiology

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List of Abbreviations

APE	atom percent excess above background level
BM	body mass(g)
BMR	basal metabolic rate
BY	between-year
CO ₂	carbon dioxide
DEE	daily energy expenditure (kJ d ⁻¹)
DLW	doubly labelled water
LMM	linear mixed effects model
LI	long interval (72h)
MR	metabolic rate
<i>R</i>	agreement repeatability
<i>R_A</i>	adjusted repeatability
SI	short interval (24h)
SS	single sample (DLW protocol)
TS	two sample (DLW protocol)
σ_{α}^2	inter-individual variation
σ_{ε}^2	intra-individual variation

1 Introduction

1.1 Repeatability of phenotypes

Within the field of animal behaviour and energetics the consistency of phenotypes are receiving greater attention than ever (Nespolo & Franco 2007; Bell, Hankison, & Laskowski 2009). Phenotypic traits which are consistent over time support the assumption that point estimates of these traits are representative for that trait of other points in time as well as representative for an individual. In biological studies point estimates are common, despite uncertainty regarding the consistency of measured traits. A violation of the aforementioned assumption may give rise to fallacious inferences when interpreting data. It is therefore of great interest to assess the consistency of traits. This task is one of variable difficulty, depending on the trait and methods available. Characteristics such as eye colour or sex usually allow for easy assessment as we simply, by experience, do not expect these to vary within the lifetime of an individual. Other traits are less easily assessed and vary over the course of a lifetime, both within and between individuals. Such traits can be categorized as plastic or labile, and examples include body mass (BM) and metabolic rate (MR) (Nussey, Wilson, & Brommer 2007). The changes that occur in plastic traits are due to either internal or external environmental variations (Price, Qvarnström, & Irwin 2003), and if reversible can be termed flexible. The manner in which a plastic trait varies can be influential for the fitness of an individual or a species. The classical thought is that if a trait varies in a consistent manner within or across species *and* has an effect on the fitness (e.g. reproductive outcome), it may be acted upon by natural selection and play a role in driving genetic evolution (Price *et al.* 2003). As such repeatability estimates may be indicative of the upper limit of heritability of a trait (Boake 1989) under certain conditions (Dohm 2002). Examples of studies of trait consistency include the running speed of lizards (Van Berkum *et al.* 1989), boldness in the field cricket (Hedrick & Kortet 2011), startle response in sea anemones (Briffa & Greenaway 2011) and basal metabolic rate (BMR) of kittiwakes (Bech, Langseth, & Gabrielsen 1999). They all share in common that several repeated measurements were obtained for the trait of interest on several individuals of the same species.

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Recent papers have outlined the need for validation studies on point estimates of energetic traits (McKechnie & Swanson 2010; Welcker *et al.* 2010), a repeated request from studies dating more than 10 years back (Speakman *et al.* 1994; Hayes, Bible, & Boone 1998). Several updated guidelines exist on how to assess the consistency of measurements (Nakagawa & Cuthill 2007; Watson & Petrie 2010; Wolak, Fairbairn, & Paulsen 2012). In general, this is done by calculating a statistic called repeatability (R). It expresses the proportion of inter-individual variation (σ_{α}^2) of the total amount of variation (eq. 1). Total variation is the sum of inter-individual variation and intra-individual variation (σ_{ε}^2). Repeatability is also commonly referred to as the intra-class correlation coefficient (ICC), where class represents groups or individuals (Sokal & Rohlf 2011).

$$R = \frac{\sigma_{\alpha}^2}{\sigma_{\alpha}^2 + \sigma_{\varepsilon}^2} \quad (1)$$

A high R indicates that measurements from different points of time agree well (i.e. high precision). Repeatability estimates also quantify the stability or consistency of the trait measured and the two terms R and consistency will in the following be used interchangeably. In the most extreme case of repeatability a difference exists between the measured individuals ($\sigma_{\alpha}^2 > 0$), but no variation exists between the repeated measurements of each individual ($\sigma_{\varepsilon}^2 = 0$), resulting in perfect repeatability of 1. Theoretically, further measurements of such a trait will convey no new information for a given individual, meaning that the point estimates are reliable. There are two caveats regarding the interpretation of this statistic. First, as equation 1 shows, R is a function of both inter σ_{α}^2 - and intra-individual variation σ_{ε}^2 . Consequently, any consistency between measurements ($R > 0$) will only occur if inter-individual variation also exist ($\sigma_{\alpha}^2 > 0$). Secondly, R can be high for two reasons, low intra-individual variation σ_{ε}^2 (relative to σ_{α}^2) or high inter-individual variation σ_{α}^2 (relative to σ_{ε}^2).

1.2 Repeatability of metabolic rates

A meta-analysis concluded that metabolic rate is significantly repeatable and not statistically affected by species, type of metabolism, time between measurements or number of individuals (Nespolo & Franco 2007). Studies on the repeatability of metabolic rates are however biased towards endotherms, with only few focusing on insects, reptiles and fish (Maciak & Konarzewski

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2010), as well as biased away from daily energy expenditure (DEE) of wild animals. A reason for the latter is perhaps that DEE must be estimated from free-ranging animals, demanding researchers to capture individuals several times to estimate repeatability. Studies on energy expenditure of wild animals under natural conditions are often done using the doubly labelled water method (DLW) (Speakman 1997). By introducing labelled water into an animal this method allows for the calculation of CO₂-production and thereby an estimation of energy expenditure. Introduction of isotopes is usually done by intra-peritoneal or intra-muscular injection. Then, following the most common procedure, the animal is held confined for a certain amount of time for equilibration of the DLW with the body water pool. Depending on body size this can last for 1-4 h (Speakman 1997). Blood-samples are then collected to estimate the turnover rates of isotopes, one initial sample after the confinement period and a final sample at recapture, after spending a certain amount of time ranging freely. The greatest benefits of this method are that it allows animals to range freely and that it is relatively non-invasive (Butler *et al.* 2004). Typically, measurement periods have been around 24h (or a multiple thereof), assuming that the animals' behaviour follows a diurnal rhythm. By obtaining samples as close as possible to 24h or a multiple thereof, researchers have attempted to avoid the large influence deviations from this time period could have on energy expenditure. However, it is also often assumed that these estimates are representative of individuals. Speakman *et al.* (1994) found a high day to day variation in DEE of the pouched mouse (*Saccostomus campestris*) when measuring DEE under this assumption, and therefore questioned the ecological relevance of such short measurement intervals. Such high day-to-day variation may result as an artefact of stochastic events especially for shorter periods of time. Their findings were supported by Berteaux *et al.* (1996) who found a relatively low repeatability of DEE ($R = 0.261$) for the meadow vole (*Microtus pennsylvanicus*) when DEE was measured for a period of 24h. If animals do not budget their activity over the course of 24h, such single measurements will not be good predictors of the average daily energy budget of an individual. These findings were later disputed by Fyhn *et al.* (2001) who found a high consistency of DEE measurements ($R_A = 0.64$) measured over the course of 24h. None of the above-mentioned studies compared measurement intervals of other lengths. To my knowledge, such comparisons have only been accomplished for energetics of the honey possum (*Tarsipes rostratus*), which showed that a longer measurement period had a positive effect on the accuracy of such measurements (Bradshaw & Bradshaw 2007). By increasing the measurement period (>24h) one may find the time period over which animals budget their energy. If DEE still varies

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substantially after adjusting the time window of measurements, one may ask whether this is related to the method itself (DLW) or whether DEE is not a repeatable phenotypic trait. In such an event DEE may rather be related to high (and un-repeatable) variation in behaviour.

1.3 Effects of DLW application

Field studies of energy expenditure usually involve the handling of wild animals. To obtain accurate or 'true' estimates of DEE in wild animals, the study individuals must necessarily be behaving naturally, as if they had not been disturbed. Energy expenditure and behaviour are explicitly linked and energetic estimates can be directly interpreted as behaviour (Potti, Moreno, & Merino 1999). By affecting behaviour one might affect the expenditure of energy which could lead to biased estimates (i.e. inaccurate). In repeatability studies the fact that measurements are repeated, and often over a short time span can also cause an effect on the estimated of energy expenditure in itself, depending on the sensitivity of the study subject and time between measurements. Dohm (2002) defined this as a negative correlation with the temporary environment. Animals might learn or acclimatize to being handled thus reacting differently from measurement to measurement, which would increase the intra-individual variation and lower repeatability.

Although the application of the DLW method is generally recognized to be low impact, effects on behaviour have been well-documented, leading to two recommended alterations in methodology (Schultner *et al.* 2010). First: by applying a variant of the DLW method that is less invasive one can reduce its impact. Briefly, the DLW method can be applied in animal energetics studies by means of two different protocols, the two sample (TS) DLW method and the single-sample (SS) DLW method (Speakman 1997). The SS protocol is less invasive because it only requires a single blood sample, which is drawn at the end of the measurement period and more importantly, no confinement period is needed after injection allowing for instant release of the animal after injection. By applying the SS protocol, and thus lowering the amount of handling and blood sampling, estimates of DEE are likely to be more accurate (Schultner *et al.* 2010). The trade-off for accuracy, however, is lower precision, since equilibrium isotope concentrations must be derived from other individuals. Second: effects of the DLW method could be reduced by changing the TS protocol in a manner which decreases the stress induced by handling. This could produce both accurate and precise estimates of DEE. One way of circumventing stress induced by

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handling could be to lower the relative amount of handling. This could be done simply by lengthening the measurement period.

1.4 Aim of study

In the present study, multiple measurements of DEE of individual kittiwakes were obtained by use of the DLW method. To quantify the consistency of DEE on a broad time scale, repeated measures were obtained over a period of four years. To test for an effect of interval length, repeated measures were obtained within breeding seasons using two different measurement intervals (24h vs. 72h). Finding the time-frame for which measurements of DEE give the highest repeatability could increase the quality of point estimates for a wild animal. Similarly, repeated measurements were taken using two different DLW protocols (SS vs. TS) to test for an effect of handling. The kittiwake was chosen as the study-species because it is relatively easy to gain access to and is a well-studied seabird within the Northern-Atlantic Arctic (Bech *et al.* 2002).

2 Material & methods

2.1 Study area & study species

The study was carried out in a colony of kittiwakes breeding on Blomstrandhalvøya in Kongsfjorden on the west coast of Svalbard (78°54'N, 12°13'E) in the European Arctic. Data were collected during the breeding seasons in 2006, 2007, 2009 and 2010. Kittiwakes are medium-sized seagulls (females approx: 350g, males approx: 400g), which spend most of their life offshore in the North Atlantic (Frederiksen *et al.* 2011). During summer they nest on cliffs near the sea, where breeding pairs usually lay 2-3 eggs, but only raise around 1-2 chicks. Both parents assist each other in the strenuous task of raising their chicks, by taking turns to attend the chick at the nest or collect food from the sea. The diet of kittiwakes varies from year to year, but consists mostly of fish and to some extent invertebrates (Barrett *et al.* 2002).

2.2 Study design & experimental procedures

To estimate CO₂ production and DEE of kittiwakes the DLW method was used (Lifson & McClintock 1966). Data from several years (4y) were collected to determine between-year repeatability. To provide data for within-season *R* birds were sampled twice per breeding season. To determine the effect of the measurement interval, birds were sampled with a 24h (short) or a 72h (long) interval in 2006/7 ($N_{2006} = 24$, $N_{2007} = 20$ individuals) and 2009/10 ($N_{2009} = 32$, $N_{2010} = 92$), respectively. To test for an effect of the handling procedure a comparison of DEE estimates using two different DLW protocols was included in this study. In 2006 and 2007, one of two treatments was randomly assigned to each kittiwake (SS : $N_{2006} = 22$, $N_{2007} = 20$, TS: $N_{2006} = 24$, $N_{2007} = 20$) (Schultner *et al.* 2010).

It has been shown that kittiwakes experience an increase in DEE throughout the chick-rearing period. In all year, measurements were therefore restrained to be performed when the chicks were ~20 days old (range 15-22). Therefore, it was also of importance that consecutive measurements (to estimate within-season *R*) were as close together in time as possible, so as not to be

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confounded by an effect of chick age (Fyhn *et al.* 2001). Birds were caught straight off their nests, using a noose attached to a long rod, and placed in cotton bags. Each individual was weighed initially using a Pesola spring balance (± 5 g) and injected with DLW using a gastight Hamilton syringe (year:dosage in mL; 2006:0.41, 2007:0.34, 2009:1.00, 2010:1.25). Birds in 2006/7 were injected into the pectoral muscle whereas birds in 2009/10 were injected intraperitoneally. The dosage of DLW contained 33.9, 41.1, 35.1 and 36.2 atom per cent excess (APE) deuterium (^2H) and 62.1, 56.8, 66.9 and 64.0 APE oxygen (^{18}O) in 2006, 07, 09 and 2010, respectively. Birds not previously captured were banded with a numbered steel band as well as a plastic band with a 3-digit letter code. Kittiwakes in the SS treatment were released immediately after injection. TS kittiwakes were kept for one hour to ensure equilibration of isotopes with the body water (Speakman 1997). TS kittiwakes were weighed before an initial blood sample was drawn. Additionally, a blood sample from 6-12 unlabelled kittiwakes was drawn in order to estimate mean background isotope enrichment (Speakman and Racey 1987; method D). The brachial vein was punctured and blood collected into four 75 μL microcapillary tubes. Using a butane torch tubes were immediately flame-sealed. Prior to release all birds were marked with red or blue marker pens on head and breast feathers to allow for easy identification. At recapture, birds were weighed again and a final blood sample was collected to estimate isotope turnover rates. In 2006 and 2007 all birds were recaptured twice after injection, at approximately 24h and 48h after release (Table 3.1, SI-TS and SI-SS). This was done to estimate R of a short (24h) measurement interval. In order to estimate R over two 3-day intervals, birds in 2010 were recaptured only once after injection at approximately 72h (Table 3.1, LI-TS). However, immediately upon recapture, birds were reinjected with a similar dose of DLW and recaptured a second time after approximately 72h. In 2009, birds were only recaptured once after approximately 54h (Table 3.1, included in BY-TS).

In all years a minimum of 93 % of all injected birds were successfully recaptured and sampled as described above.

2.3 Lab procedures & DEE calculations

Isotopic enrichments of blood samples from all study years were determined by isotope ratio mass spectrometry as described in Speakman *et al.* (1990) for oxygen (^{18}O) and Speakman and Król (2005) for deuterium (^2H). Blood samples were vacuum distilled into glass Pasteur pipettes (Nagy 1983), and the water obtained was used for isotope ratio mass spectrometry. For analysis

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of ^{18}O the water was equilibrated with CO_2 with a known oxygen isotopic enrichment and the resultant $^{18}\text{O}:^{16}\text{O}$ ratio was analysed in an isotope ratio mass spectrometer (IRMS). The ^2H was analysed by injecting the samples into a heated ($170^\circ\text{-}180^\circ\text{C}$) injector. The resultant water vapour was reduced to hydrogen gas and carried by carrier helium gas-stream into a single-inlet IRMS for analysis of the $^2\text{H}:^1\text{H}$ ratio. Each water sample was sub sampled twice and the calculated average was used in the further DEE calculations. Background samples from each year were similarly analysed and their average subtracted from initial and final isotopic enrichments in order to correct for the natural background levels of ^{18}O and ^2H (Speakman & Racey 1987; method D). To estimate the enrichment of the injectate, the original injectate from each year was diluted with tap water to make a dilution series. Each dilute was then sub sampled 5 times before mass spectrometric analysis of ^{18}O and ^2H . Results from the 5 subsamples were averaged for each of the dilutions and then again averaged over the range of the dilution series (Speakman 1997).

As recommended for birds with a body mass less than 1 kg (Speakman 1993) a single pool model was used to calculate CO_2 -production ($\text{ml CO}_2 \text{ h}^{-1}$) (equation 7.17 Speakman, 1997)(Appendix I). The fixed evaporative water loss of 25%, assumed in this equation, has been validated for bird studies (Visser & Schekkerman 1999; Van Trigt *et al.* 2002). For TS birds, initial dilution spaces were calculated by the plateau method (Halliday & Miller 1977) and used to estimate the actual amount of total body water (g) as well as the percentage of body water. By assuming that percentage of body water did not change throughout the measurement interval, final dilution spaces were inferred from the initial dilution space by multiplying the final body mass by the ratio of the initial amount of body water to initial body mass (Speakman 1997). Only the final blood sample was obtained from SS birds and initial isotope enrichment was therefore estimated using the relationship of initial isotope enrichment and body mass established for TS kittiwakes during the field seasons of 2006 and 2007 (Schultner *et al.* 2010). Estimates of CO_2 production in 2006 and 2007 were converted to energy equivalents using year specific conversion factors, based on the known diet composition of the birds (Schultner *et al.* 2010). Estimates in 2009 and 2010 were converted using the mean conversion factor derived from year specific factors estimated over 5 study years (Welcker *et al.* 2010). This could be done because variation among year specific conversion factors was negligible over the years (CV: 0.079%) (Welcker *et al.* 2010).

2.4 Datasets & statistics

Data from ~5% of birds were removed because final enrichment was too close to background or capillaries were not completely sealed. For comparison of R estimates from different measurement intervals (short interval: 24h and long interval: 72h) and different DLW protocols (SS and TS) three datasets were compiled, in which each bird had > 2 estimates (Table 1). These three datasets were all in the category *within-season R*. The short interval datasets consisted of DEE estimates from the two adjacent 24h periods, one dataset for TS birds (short interval two sample, SI-TS) and one for SS (short interval single sample: SI-SS) birds (2006 and 07). The long interval (LI-TS) data set consisted of estimates from the two adjacent 72h periods derived from re-injected birds (measured in 2010). For estimates within the category *between-year R* a final data set was compiled consisting of DEE estimates from all four study years (TS method only). For this dataset DEE was estimated over the complete measurement period (i.e. injection to second recapture) in 2006 and 2007, and contained only the first estimate from re-injected birds in 2010 (*between-year*: BY-TS). An overview of the four datasets can be seen in table 3.1.

Repeatability was calculated based on linear mixed-effects models (LMMs). Variance components were estimated by restricted maximum likelihood (REML). Bird identity was included as a random factor, thus adding a random intercept for each bird. In the simplest model (eq. 2) it was assumed that no systematic difference existed between repeated measures and that the birds' true energy expending abilities did not change across repeated estimates. In this manner, any variance across repeated measures can be considered as random measurement error or, in our case as an expression for intra-individual phenotypic flexibility including any 'true' measurement error:

$$y_{ij} = \beta_0 + \alpha_i + \varepsilon_{ij}, \quad (2)$$

where y_{ij} is DEE measurements for i th individual at the j th occasion. β_0 is the grand mean/population intercept. The estimated variance components from the model are α_i , which in relation to eq. 1 is inter-individual variation (σ_α^2), and ε_{ij} is intra-individual variation (σ_ε^2). Repeatability was calculated as the intra-class correlation coefficient for single estimates (ICC)

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(Lessells & Boag 1987)(eq.1). This is a variant of repeatability, here termed *agreement repeatability* or simply R (Nakagawa & Schielzeth 2010).

Mixed models were fitted with fixed effects (eq. 2) to determine the impact of fixed effects on the variance components associated with the random factors. By adjusting for covariates or factors such as body mass and year another variant of repeatability was calculated, termed *adjusted repeatability* R_A . To calculate R_A adjusted for body mass, ‘body mass’ was added as a fixed effect in the mixed model. By adding body mass it is possible to account for variation in DEE due to differences in body mass. Adding body mass also accounts for sex differences, since the kittiwake is a sexually size-dimorphic bird with males weighing more than females (Welcker *et al.* 2010). The effect of a continuous fixed factor such as body mass, is defined by the slope of the model, which in our case expresses how the mean DEE differs in relation to body mass. To control for potential effects of variable environmental conditions between the different study years, ‘year’ was added as a categorical fixed factor in the model for *between-year* R (year-adjusted R). To account for potential systematic differences between the first and the second measurement period within each season, ‘measurement period’ was added as a fixed factor for *within-season* models (period-adjusted R). The effect of a categorical fixed factor is defined by differences from the overall mean for each level of the factor.

A previous study on data from the same colony (including data from 2006/7) showed that parent kittiwakes raising two chicks had similar rate of DEE as those of parents raising singletons (Welcker *et al.* 2010). This has also been found for other birds (Potti *et al.* 1999). For this reason brood size was not considered as factor in the model. The continuous daylight in the arctic summer enables kittiwakes to raise their chicks around the clock without a diurnal rhythm. For this reason no deviations from a 24h (or a multiple thereof) recapture window were taken into account. Estimates of R were considered statistically significantly different from zero when the 95 % confidence intervals (CI's) did not include zero. Models were checked for constant variance of the residuals and approximate normality of the predicted random effects. All LMMs were fit using the package lme4 in R.2.15 (Bates, Maechler, & Bolker 2011; R Development Core Team 2012). Agreement and adjusted repeatabilities with CI's (parametric bootstrapping 10000 permutations) were calculated using the package rptR (Schielzeth & Nakagawa 2011).

3 Results

When comparing estimates from different years, thus looking at *between-year* R , repeatability was moderate to high ($R = 0.430$, Figure 3.1, Table 3.2). *Within-season* estimates of R differed considerably, depending mostly on the length of the interval from injection to recapture, and somewhat on which DLW protocol was applied. The long interval measurements resulted in a substantially higher repeatability ($R = 0.772$, Figure 3.1, Table 3.2) than the short interval, regardless of whether the TS protocol ($R = 0.108$, Figure 3.1, Table 3.2) or the SS protocol ($R = 0.195$, Figure 3.1, Table 3.2) was applied for the short interval measurements. The SS protocol resulted in a slightly higher R than the TS protocol (Table 3.2). Adjusting for body mass as a fixed factor allowed for calculation of mass-adjusted repeatability R_A . Body mass was added to all models. The effect observed for this continuous predictor variable was a decrease in the estimates of R for all categories (Figure 3.1). This was mainly due to a decrease in the inter-individual variation σ_α^2 (all models) as well as an increasing effect in some models on intra-individual variation σ_ϵ^2 (LI-TS and SI-SS) (Table 3.2). For one model (SI-TS) the variability between individuals σ_α^2 was not larger than one could expect from random variation and was fitted as zero (resulting in an estimated R of 0) when adding body mass as a factor (Table 3.2). Adding year as a fixed factor to account for differences between study years slightly decreased *between-year* R (*year-adjusted* R_A). Adjusting the estimate of R for differences between the first and second measurement period slightly increased all estimates of *within-season* R (Table 3.2).

Results

Table 3.1 Data from four datasets, *between-year* (BY-TS) and *within-season* in three different variants: two-sample protocol, long interval (LI-TS), short-interval (SI-TS) and single-sample protocol, short interval (SI-SS). Measurement period (h) for each dataset (mean \pm SD). Number of individuals $n(i)$ and total number of measurements $n(o)$. Number of repeated estimates of DEE per bird for each dataset.

Abbreviation	Category	Interval	Δh mean \pm SD	Protocol	$n(i), n(o)$	number of repeats		
						2x	3x	4x
BY-TS	Between year	LI	58 ± 10	TS	25, 56	20	4	1
LI-TS	Within year	LI	64 ± 12	TS	23, 46	23		
SI-TS	Within year	SI	27 ± 7	TS	26, 52	26		
SI-SS	Within year	SI	26 ± 4	SS	35, 70	35		

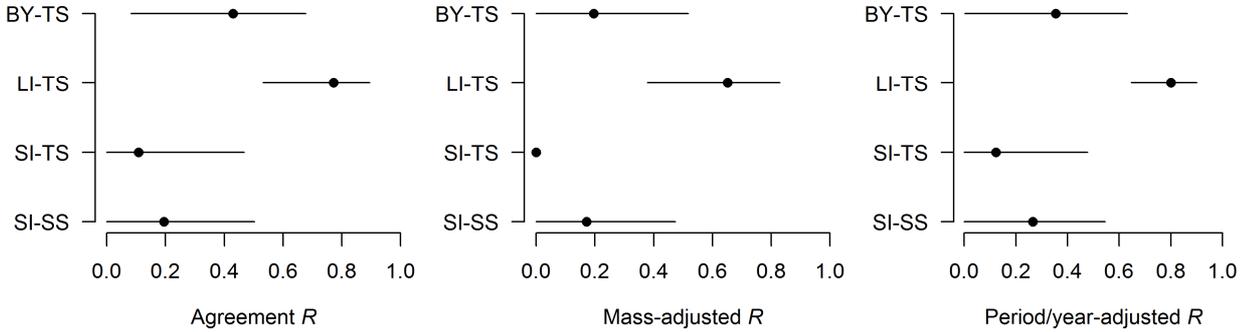


Figure 3.1 Point estimates of repeatability (R) for different categories. The line represents 95% confidence intervals found by parametric bootstrapping (10000 permutations). Confidence intervals including 0 indicate that R is not significantly different from 0. *Between-year* (BY-TS), and *within-season* (LI-TS), (SI-TS) and (SI-SS). LI = long interval, SI = short interval, TS = two sample doubly labelled water (DLW) protocol, SS = single sample DLW protocol. Agreement R = models excluding any fixed factors. Mass-adjusted R = models with mass as a fixed factor. Period/year-adjusted = models with period (*within-season* R) and year (*between-year* R) as fixed factor. The point estimate for SI-TS for mass-adjusted R was exactly zero and no confidence intervals was calculated.

Results

Table 3.2 Variance components from mixed models and repeatability (R) estimates of DEE for all categories. *Between-year* (BY-TS), and *within-season* (LI-TS), (SI-TS) and (SI-SS). LI = long interval, SI = short interval, TS = two sample doubly labelled water (DLW) protocol, SS = single sample DLW protocol. Agreement R = models excluding any fixed factors. Mass-adjusted R_A = models with mass as a fixed factor. Period/year-adjusted R_A = models with period (*within-season R*) and year (*between-year R*) as fixed factor. Uncertainty estimates (95 % confidence interval) derived from parametric bootstrapping (10000 permutations).

Abbreviation	n(i), n(o)	Agreement R			Mass-adjusted R			Period/year-adjusted R		
		R	σ_a^2	σ_ε^2	R_A	σ_a^2	σ_ε^2	R_A	σ_a^2	σ_ε^2
BY-TS	25, 56	0.430 (0.082, 0.677)	25767	40330	0.196 (0, 0.518)	7835	32064	0.354 (0.002, 0.631)	18664	34025
LI-TS	23, 46	0.772 (0.532, 0.895)	46232	13662	0.652 (0.378, 0.829)	26306	14050	0.8 (0.647, 0.9)	47173	11781
SI-TS	26, 52	0.108 (0, 0.468)	7185	59047	0 (0*)	0	54661	0.123 (0, 0.478)	8282	59047
SI-SS	35, 70	0.195 (0, 0.503)	13001	53795	0.172 (0, 0.474)	11346	54536	0.265 (0, 0.546)	16740	46317

* The point estimate for SI-TS was exactly zero whend adjusting for body mass and no confidence interval was calculated.

4 Discussion

The present study examined whether estimates of DEE of breeding kittiwakes were repeatable when applying differing measurement intervals and DLW protocols both within a season and between years. Repeatability represents the proportion of the overall variation observed in a trait in relation to variation between individuals. The results demonstrate that: (i) DEE is a repeatable trait both over several years and within seasons, (ii) within-season R is highest when using a longer measurement interval and (iii) applying the less-invasive SS-protocol had a negligible effect on the R of short interval measurements. Adjusting for body mass had mainly an effect on the variation between individuals, thus decreasing R for most categories except for long interval measurements taken within a single season (TS birds). The remaining intra-individual variation was not explained by differences in body mass suggesting this was due to other factors not in the model, such as age or type of activity the bird was engaged in during measurements.

4.1 Between-year R

Between years, R was moderate ($R = 0.430$) between years indicating that individual kittiwakes to some extent were consistently different from one another in their energy expenditure over longer periods of time. A study by Fletcher *et al.* (2012) measured DEE of free-ranging red squirrels (*Tamiasciurus hudsonicus*) over several years also using the TS DLW protocol and a measurement interval > 2 d. They found however no evidence that individual differences influenced the variation in DEE and concluded that DEE was not a repeatable trait in that specific population. Their estimates were obtained from different seasons/reproductive stages and even after adjusting for these factors they found no consistent individual differences. This might be true also for seabirds, meaning that DEE estimates from a specific period such as breeding do not represent other periods, such as the overwintering stages of kittiwakes. The migratory life of many seabirds complicates the acquirement of energetic measurements from other stages than the breeding season, the phase in which they are the most associated to land and hence very little energetic data beyond the breeding season exists. By using the heart rate method on two seabird species it has been shown, not surprisingly, that DEE varies notably throughout the annual cycle and that much of this variation can be ascribed to differing activities such as migration and

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breeding (Green *et al.* 2009; White *et al.* 2011b). Yet they did not investigate whether individuals varied in a consistent manner and this should therefore not occlude the result from the present study that individuals appear to be consistently different over several breeding seasons. This is the first study to report R of DEE over such a long time scale, which is not surprising given the scarce amount of literature dealing with R of DEE, however only few individuals were sampled more than twice (i.e. > 2y). Long-term studies of MRs in wild animals have previously focused on BMR (Broggi *et al.* 2009; Bushuev, Kerimov, & Ivankina 2011). Most notably was a study which in accordance with the present study estimated the R of the BMR female kittiwakes to be similar between seasons ($R = 0.347-0.520$) (Bech *et al.* 1999). The fact that both BMR and DEE of kittiwakes appear to be consistent over longer time periods indicate that kittiwakes might operate under specific 'metabolic' strategies. Individuals with high DEE might have a high BMR as has been shown for the Atlantic salmon (*Salmo salar*) (Millidine, Armstrong, & Metcalfe 2009). A recent interest in linking metabolism and behaviour in relations to inter-individual variation within species has resulted in evidence of varying strength (Biro & Stamps 2010; Lantová *et al.* 2011). Interspecifically such strategies have also been related to latitudinal gradients (e.g. temperature and daylight) (Anderson & Jetz 2005)

4.2 Measurement interval

The results for between-year R were obtained using a long measurement interval and suggest that estimates from single years to some extent are representative of estimates the following years during the breeding season. Other repeatabilities of standardized MRs such as BMR has in general been found to be high (Versteegh *et al.* 2008, and references therein; Nilsson, Akesson, & Nilsson 2009; Chappell *et al.* 2011), and has in some studies been found to be higher within-season than between season (Rønning, Moe, & Bech 2005; Broggi *et al.* 2009). This pattern resembles that of the present study which shows that within-season R of DEE is higher than between-year R . This was however only true for estimates obtained using the long measurement interval. The short interval resulted in very low R with confidence intervals including zero. Two studies on captive wild rodents, presented negative correlations between day-to-day measurements of DEE (Speakman *et al.* 1994) and a low R (Berteaux *et al.* 1996). It was suggested that the source of this variation was a lack of balance in the energy budgets of a time-scale of a single day. The fact that only the long interval resulted in a high R in the present study supports the assumption put forward first by Speakman *et al.* (1994), that the average DEE of an

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animal, here the kittiwake, is not necessarily obtainable from a single 24h measurement. High variation in repeated estimates from individuals (i.e. high intra-individual variation σ_{ϵ}^2) points to the fact that such estimates, are more likely to simply represent an individual's specific behaviour at the time of measurement and that they most likely were doing different things from day to day (Fletcher *et al.* 2012). Such stochastic day-to-day variation could disguise any existing consistent differences between individuals in an analysis of R . By extending the measurement interval in this study intra-individual variation was reduced drastically in relation to inter-individual variation and thus resulted in a high R . From this it follows that kittiwakes seem to be consistently different in DEE during late breeding.

Fyhn *et al.* (2001) found however in 1997 and 1998 a similarly high R ($R = 0.64$) for DEE of kittiwakes using the short interval measurement as the present study did for long interval measurements. This contradicts the findings from this study and points towards the fact that it might not only be the measurement interval itself which produces unreliable estimates. More importantly perhaps, is the fact that approximately half of the measurements done in 1997 and 1998 were done on parent kittiwakes raising chicks at the age of 5-7 days, whereas the remaining half were done in late chick-rearing (chick age 20-27d) (Fyhn *et al.* 2001). Parents attending smaller chicks seem to follow a tighter schedule (thus reducing stochastic day-to-day variation) and their behaviour might also to a lesser degree be affected by handling (Gabrielsen, Klaassen, & Mehlum 1992). This could translate into a lower intra-individual variation in DEE and a higher R when using the short interval for DLW measurements. The different timing of measurements in the two studies in addition to different statistical procedures complicates direct comparison, but they might suggest that the 24h measurement interval can be applied usefully in early but not in late chick-rearing for obtaining average DEE estimates.

4.3 Stress by handling- period-adjusted R

It has been shown that the TS-DLW protocol can change the natural behaviour of kittiwakes thereby biasing DEE estimates (Jodice *et al.* 2003; Schultner *et al.* 2010). Kittiwakes treated with TS DLW protocol returned later to their nests after release and had overall a reduced nest attendance compared to kittiwakes treated with SS DLW and a control group (Schultner *et al.* 2010). This resulted in a decreased DEE compared to SS birds, which was explained by altered behaviour when birds were at sea. Furthermore this effect was stronger in so called 'bad years' (e.g. 2006), which were characterized by a poor diet (less fish) and a lower reproductive success.

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This led to the suggestion that stress induced by the DLW protocol was additive to external stressors (Welcker *et al.* 2010; Schultner *et al.* 2010). If truly additive and of uniform magnitude for each handling incidence, such stress should in theory not decrease R more in bad years compared to better years. It however appears that the effect of handling diminishes with each repeated handling, suggesting that individuals are conditioned by the repeated handling in itself and thus respond systematically different from one measurement to another (Fyhn *et al.* 2001; Schultner *et al.* 2010). If the temporary environment associated with each repeated measurement is correlated this might underestimate R (Dohm 2002). By adding period as fixed factor into the model, thus trying to account for the contribution of each period as a unique temporal environment, R increased slightly. Period as a factor did in general not explain much of the variation in DEE, indicating that other factors not included were stronger contributors to the variation within and across individuals. Even when near-natural behaviour was obtained by using the SS DLW protocol, thus ruling out any potential bias caused by handling (Schultner *et al.* 2010), R was still very low when using the short interval measurements. This provides strong evidence against the assumption that measurements of an interval of 24h represent the average DEE of kittiwakes, at least in late chick-rearing.

4.4 Body mass- & year- adjusted R

Year as a factor in *between-year* R did not explain variation in DEE very well. As it turns out DEE across years is strikingly similar in kittiwakes, leaving little variation to be explained (Welcker *et al.* 2010). The mass-adjusted R 's reported here are rather low (0 -0.196; except for LI-TS: 0.652) compared to mass-adjusted R 's in kittiwakes of DEE (0.64; Fyhn *et al.* 2001) and BMR reported elsewhere (0.35-0.52 Bech *et al.* 1999). By adding body mass as a factor the variance between individuals decreased while the variance within individuals remained largely unchanged. This means that differences in DEE across individuals could to a certain degree be explained by differences in body mass between individual kittiwakes. Body mass is a continuous predictor variable that varies on two hierarchical levels, both inter and intra-individually, were the inter-individual level will tend to decrease R and the intra-individual level will tend to increase R (Pol & Wright 2009; Nakagawa & Schielzeth 2010). The R of MR will thus often hinge on the R of body mass per se, and as such it would be wise to investigate both simultaneously (Szafrńska, Zub, & Konarzewski 2007b; Broggi *et al.* 2009). Body mass has a large effect on MR if looked at across species (Bryant 1997; Nagy 2005), but within species body mass usually has a much lower

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effect (White 2011a). Differences in the R of whole-animal MR and body mass-corrected MR, have been widely observed, with the latter tending to be the lower rate (Konarzewski, Ksiazek, & Lapo 2005). Mass-corrected MR can be derived in two ways, either by division of MR by body mass, which is called mass-specific MR, or by a regression of MR on body mass (mass-independent MR) or as incorporation into a mixed model as a fixed factor. Either way the discrepancy between repeatabilities of mass-corrected MR and its corresponding unadjusted MR could also lie in the fact that random fluctuations of body mass can occur, due to for example recent feeding or urination, despite precise measurements (Konarzewski *et al.* 2005).

4.5 DEE as a phenotypic trait

Extending the measurement interval appeared to provide more reliable point estimates of DEE, which is of great importance when wanting to relate DEE to other measurements or observations of phenotypic quality or life-history traits (Hörak *et al.* 2002). The moderate to high R 's reported here using the long interval suggests that kittiwakes appear to be relatively consistent over both a short and long term scale. The SS-protocol appeared also to provide reliable measurements of DEE, in the sense that they reflected what the individual kittiwake was doing at the time of measurement (see further down) (Schultner *et al.* 2010), but could not serve as a good proxy for the average DEE of kittiwakes considering the low R found. Under such circumstances it appears that DEE estimates are not of a phenotypic trait, but rather a snapshot of an individual birds DEE. By reducing handling a near-natural behaviour was observed, something that is essential for reliable DEE estimates. Another way of decreasing stress induced by handling was attempted by decreasing the relative amount of stress by a longer measurement interval. A way of quantifying this effect could be by analysing other variables such as behaviour and relating these to DEE from measurements using different intervals as well as relating to a control-group. Fyhn *et al.* (2001) found a positive correlation between nest attendance and FMR ($r^2 = 0.50$), similar to what Schultner *et al.* (2010) found for SS birds in 2006 and 2007 ($r^2=0.45$), but not for TS birds ($r^2=0.02$) when using the short interval. If R is high and correlated to responses in other traits measurements of this trait become interesting when speaking of natural selection. Consistent individual differences in DEE might translate into consistent differences in behaviour (Biro & Stamps 2010). Not all repeatable traits can serve as proxies for phenotype, for instance, a study by David *et al.* (2012) measured the breathing as a proxy of stress in the zebra finch. The 'trait' was found to be repeatable but not a good indicator of personality as it was not related to any

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other behavioural traits. It is however possible to imagine that birds which generally exhibit a high DEE may generally spend more time on energetically costly activities such as flying (Jodice *et al.* 2003), thus portraying an active phenotype with DEE and behaviour as part of a behavioural syndrome (Sih *et al.* 2004). Resting MR (similar to BMR) has been found to correlate positively with behaviour such as aggressiveness for many different taxa (Biro & Stamps 2010). If such correlations also are found in relation to life-history traits, such as mortality or reproductive success, MRs could be a factor contributing to the evolution of phenotypes. Not much is known regarding the association between fitness and metabolism and the few studies existing on this subject (BMR) have all resulted in ambiguous results, which casts doubt on the role MRs might have on individual fitness (Boratyński & Koteja 2009 and references therein; see also Pemberton 2010). One important pathway of how DEE is supposed to be related with fitness is the ‘free radical damage hypothesis’ - higher metabolism results in higher production of free radicals and therefore in cellular damage resulting in earlier death (Beckam & Ames 1998). A condition for this influential hypothesis is that individuals differ consistently in their DEE and that their ‘metabolic strategy’ is heritable. It has yet to be demonstrated any negative correlations between DEE and for example the probability of returning to the colony the following year (Welcker *et al.* 2010), which may be routed in the fact that measurements of DEE are unreplicable of the individual kittiwake (when using a short interval).

Studies on R can be useful as indicators for future studies of heritability by setting an upper limit (Falconer & Mackay 1996). Heritability might however be lower than R if the repeated measurements are correlated not only genetically but environmentally as well (Visscher, Hill, & Wray 2008). In addition to the necessity of individual variation, a trait must be heritable to be acted on by natural selection. Positive (i.e. significantly different from zero) heritability has been shown for RMR and BMR in several passerine species (Rønning *et al.* 2007; Nilsson *et al.* 2009; Bushuev *et al.* 2011) as well as for mammals (Szafrńska, Karol, & Konarzewski 2007a; Careau *et al.* 2011), suggesting that further studies on the heritability of DEE or BMR in seabirds might well be fruitful, especially when seen in the light of the present study, and the study on R of BMR in kittiwakes by Bech *et al.* (1999).

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Appendix I

A walk-about DEE calculations

– freely from Speakman 1997.

The procedure of calculating CO₂ production using the DLW method (DLW) is described step by step according to Speakman, 1997. This estimate is used to calculate daily energy expenditure. For a more detailed description, in-depth explanations of assumptions and alternative models see Speakman (1997).

Required data: initial and final isotope enrichment of blood samples and time elapsed between sampling as well as body mass at each blood sampling and background isotope enrichment. As an example I have chosen the kittiwake BAH, which was twice injected in 2010.

The calculations resulting in CO₂ production involves seven steps. The first two steps were in our case done by the lab and were composed of estimating the injectate enrichment and converting all isotope values into p.p.m. (to allow for assessments of ratios).

Step three: first isotope turnover rates are calculated for each isotope, oxygen-18 (¹⁸O) and deuterium (²H). Turnover rate is here expressed as change in isotope (p.p.m) over time (h)

For oxygen:

$$k_o = \frac{[\log_e(\text{initial } ^{18}\text{O} - \text{background } ^{18}\text{O}) - \log_e(\text{final } ^{18}\text{O} - \text{background } ^{18}\text{O})]}{\text{time}}$$

And for hydrogen:

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$$k_d = \frac{[\log_e(\text{initial } ^2\text{H} - \text{background } ^2\text{H}) - \log_e(\text{final } ^2\text{H} - \text{background } ^2\text{H})]}{\text{time}}$$

Substituting for data from BAH:

$$k_o = \frac{[\log_e(5875.22 - 1993.23) - \log_e(2374.39 - 1993.23)]}{65.13} = 0.036 \text{ ppm}^{-h}$$

$$k_d = \frac{[\log_e(2242.52 - 156.17) - \log_e(487.27 - 156.17)]}{65.13} = 0.028 \text{ ppm}^{-h}$$

We can now calculate the k_o/k_d value to check that things are proceeding correctly

$$\frac{k_d}{k_o} = \frac{0.028 \text{ ppm } ^2\text{H}^{-h}}{0.036 \text{ ppm } ^{18}\text{O}^{-h}} = 0.79 \frac{\text{ppm } ^2\text{H}}{\text{ppm } ^{18}\text{O}}$$

Which means that almost 80 % of the oxygen loss from the body can be linked to hydrogen turnover. The range for k_d/k_o 0.5-1. A k_d/k_o smaller than 0.5 is not impossible but it implies a massive oxygen turnover relative to hydrogen, if this value exceeds 0.9 the DLW technique will probably not provide useful estimate of CO_2 production because the oxygen turnover only slightly exceeds the hydrogen turnover.

Step four: estimating the isotope dilution spaces. The dilution space is the volume of water in which the DLW is diluted (i.e. when we inject a bird). This is estimated from the extent of dilution of the DLW (obtained from a blood sample 1 hour later). These isotope spaces are then used to calculate the actual amount of water (g) in the bird (Total body water TBW) as well the percentage of body water of total body mass. We assume that the equilibration of isotope was completed exactly at the time of our initial blood sample was taken (equilibration sample). This approach is called the plateau approach (i.e. a plateau (equilibration) is reached at sampling time).

$$N_o \text{ or } N_d = \text{Mol}_{inj} \frac{E_{mix} - E_{inj}}{E_{wat} - E_{mix}} = \text{Mol}_{wat}$$

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Mol_{inj} is moles of injectate injected into the animal

E_{mix} is the equilibrium enrichment of the isotopes in the body (from initial sample)

E_{wat} is the background level of the isotopes in the body

E_{inj} is the estimate of the injectate enrichment

Mol_{wat} is the unknown value and is the equivalent moles of water in the body *if* all the exchangeable isotope in the body for either hydrogen or oxygen existed as water. Here substituting for ^{18}O first and then for deuterium:

$$N_o = \frac{0.062(5875.22-640043.6)}{1993.23-5875.22} = 10.18 \text{ mol . which is equivalent to}$$

$$TBW_{initail} = 10.18 \text{ mol} \left(2 \frac{1\text{gram}}{\text{mol}^2\text{H}} + \frac{16\text{gram}}{\text{mol}^{18}\text{O}} \right) = 183.24 \text{ g of body water}$$

$$N_d = \frac{0.062(2242.52-362162.4)}{156.17-2242.52} = 10.75 \text{ mol. which is equivalent to.}$$

$$TBW_{initail} = 10.75 \text{ mol} \left(2 \frac{1\text{gram}}{\text{mol}^2\text{H}} + \frac{16\text{gram}}{\text{mol}^{18}\text{O}} \right) = 193.5 \text{ g body water}$$

It is now useful to look at the oxygen pool size as a percentage of body mass. This bird, BAH, weighed initially 375g, which means that its oxygen pool size expressed as a percentage of body mass is:

$$BW_{Oxygen} = \frac{183.24\text{gH}_2\text{O}}{373\text{g}} * 100\% = 49.14 \% \text{ H}_2\text{O}.$$

The usual range is from about 50 to 75 %, so this is perhaps a bit on the low-side indicating that the bird is somewhat obese (more fat than water) or that the sample was contaminated during the gas preparation phase or probably more likely that isotopes leaked out during injection.

Step five: Now we can calculate the dilutions space ratio:

$$\frac{N_d}{N_o} = \frac{10.18}{10.72} = 1.06$$

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Which is within the acceptable range of 0.97-1.1 (p. 308).

Step six: estimating the final and average pool sizes using the percentage mass approach. Since we didn't kill and desiccate all injected birds to establish their final pool size (e.g. Speakman & Król 2005), we will need to estimate it. The values of the initial pool sizes as percentages of the initial body mass are applied to the final body mass to estimate the final pool sizes. One could say that we back calculate from step 4 using the final body mass. When doing this we assume that the percentage of body water is constant regardless of weight over the course of our study. We then use these pool sizes to calculate average pool size over the duration of the experiment. Here exemplified for oxygen.

$$TBW_{recapture} = BW_{Oxygen} \left(\frac{Final\ body\ mass}{100} \right), \text{ and entering into the next formula:}$$

$$N_{O\ recapture} = TBW_{recap} * 18.002\ g/mol, \text{ giving an average pool size of:}$$

$$TBW_{mean} = \frac{TBW_{recapture} + TBW_{initial}}{2}$$

Step seven: finally we can estimate CO₂ by use of the parameters above. Speakman lists a long array of different equations to use for this. The recommended equation is made by Lifson & McClintock (1966, equation 35) and utilizes as an estimate the body water pool N . Thus N = the average of N_o measured initially and the inferred final dilution space.

$$rCO_2 = \frac{N}{2.08} (k_o - k_d) - 0.015k_d N$$

Speakman has revised this equations several times and the latest recommendation is here exemplified with the previous parameters. It assumes 25% fractionated water loss.

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$$rCO_2 = \frac{9.84 \text{ mol}}{2.08} (0.036 \text{ ppm}^{-h} - 0.028 \text{ ppm}^{-h}) - 0.015 * 0.028 \text{ ppm}^{-h} * 9.84 \text{ mol}$$

$$= 0.033 \frac{\text{mol}}{h}$$

Converting to mL/h,

$$\left(\frac{0.033 \text{ mol } CO_2}{h} \right) = \left(\frac{44.01 \text{ g } CO_2}{\text{mol } CO_2} \right) \left(\frac{\text{mL } CO_2}{0.001977 \text{ g } CO_2} \right) = 737 \frac{\text{mL } CO_2}{h}$$

Converting CO₂ production into energetic equivalents using a conversion factor of 27.639 J/mL CO₂ (Welcker *et al.* 2010):

$$\left(\frac{737 \text{ mL } CO_2}{h} \right) \left(\frac{27.639 \text{ J}}{\text{mL } CO_2} \right) = 20396.5 \frac{\text{J}}{h}$$

And lastly converting into daily energy expenditure,

$$\left(\frac{20396.5 \text{ J}}{h} \right) \left(\frac{24 \text{ h}}{\text{day}} \right) \left(\frac{\text{kJ}}{1000 \text{ J}} \right) = 489.5 \frac{\text{kJ}}{\text{day}}$$

Leaving us to conclude that the kittiwake BAH in the summer of 2010 spent on average 489.5 kilojoules a day during our measurement period (24/7 – 27/7, 65h in total).

This bird was reinjected at recapture leaving us a new set of initial and final isotope enrichments from blood samples. Isotope turnover rates were estimated using the same background enrichment as for the first injection, but the isotope dilution space was estimated using the first final sample as background. This calculation led to an estimate of 769.37kJ/day in the following period (51.9h in total).

Appendix II

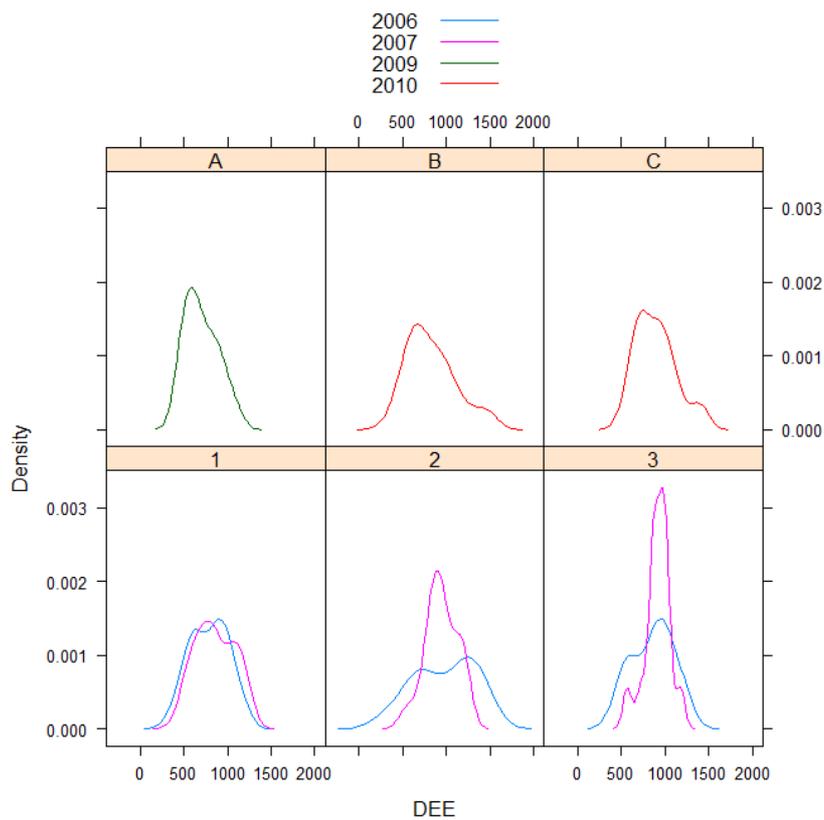


Figure 7.1: Density plot showing the individual variation of daily energy expenditure (DEE) for each different period, expressed as kJ/day. A, B, C and 3 are long interval measurements (72 h), whereas 1 and 2 are short term measurements (24h). Colour of line indicates in which year the measurements were done.

Appendix II

Histograms

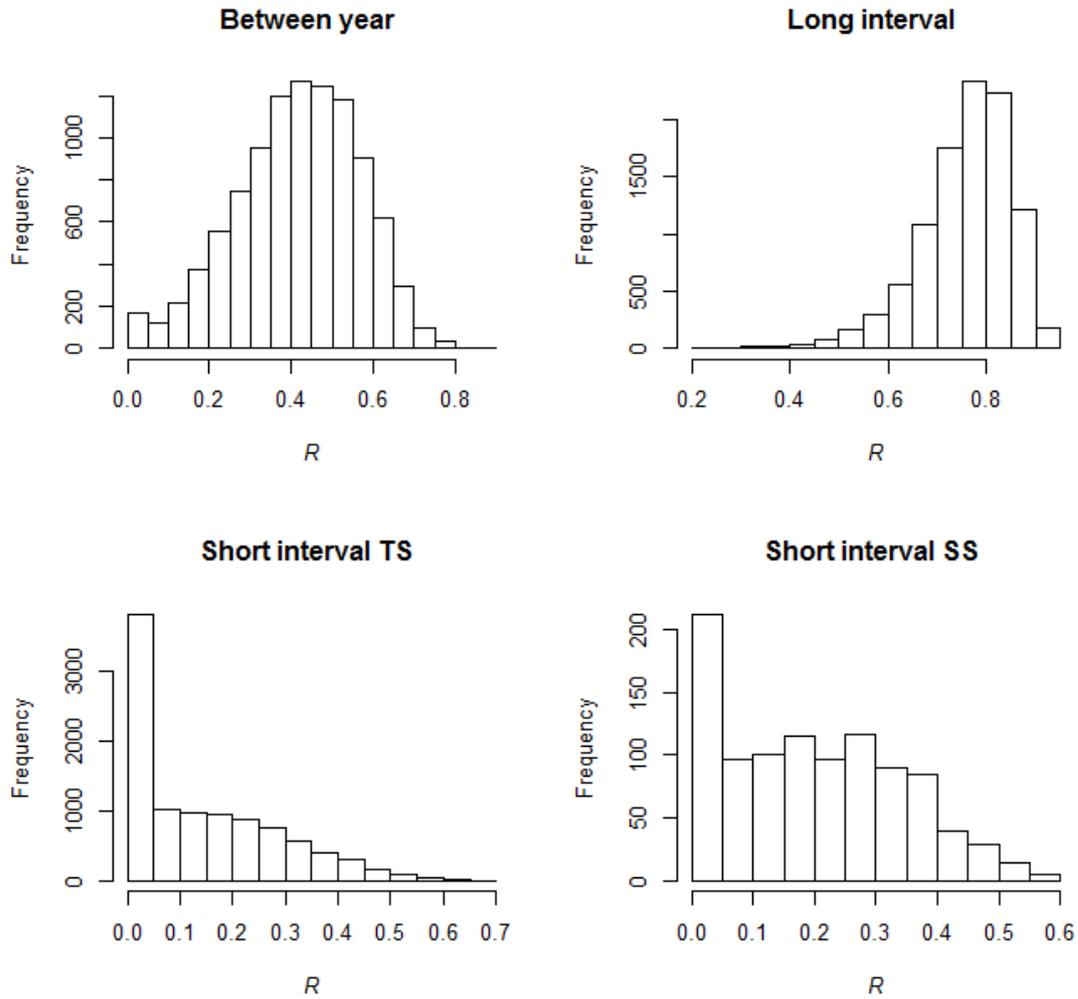


Figure 7.2: Histogram of bootstrap permutation on the statistic R for estimation of a confidence interval for each model. When bootstrapping is performed on a ratio confidence intervals can be asymmetric.

