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Application of the two-sample doubly labelled water method alters behaviour and affects estimates of energy expenditure in black-legged kittiwakes

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SUMMARY

Despite the widespread use of the doubly labelled water (DLW) method in energetic studies of free-ranging animals, effects of the method on study animals are rarely assessed. We studied behavioural effects of two alternative DLW protocols. During two consecutive breeding seasons, 42 parent black-legged kittiwakes received either the commonly used two-sample (TS) or the less invasive single-sample (SS) DLW treatment. A third group served as a non-treated control. We evaluated the effect of treatment with respect to the time birds took to return to their nest after treatment and recaptures, and the nest attendance during DLW measurement periods. We found that TS kittiwakes took on average 20 times longer to return to their nest than SS kittiwakes after initial treatment, and nest attendance was reduced by about 40% relative to control birds. In contrast, nest attendance did not differ between control and SS kittiwakes. Estimates of energy expenditure of SS kittiwakes exceeded those of TS kittiwakes by 15%. This difference was probably caused by TS birds remaining inactive for extended time periods while at sea. Our results demonstrate that the common assumption that the TS DLW method has little impact on the behaviour of study subjects is in some circumstances fallacious. Estimates of energy expenditure derived by the SS approach may thus more accurately reflect unbiased rates of energy expenditure. However, the choice of protocol may be a trade-off between their impact on behaviour, and hence accuracy, and their differences in precision. Adopting procedures that minimize the impact of TS protocols may be useful.

Key words: DLW, single sample, behavioural effect, nest attendance, field metabolic rate, *Rissa tridactyla.*

INTRODUCTION

The doubly labelled water (DLW) method (Lifson and McClintock, 1966; Nagy, 1980; Speakman, 1997) is the current standard technique to estimate the energy expenditure of free-ranging animals (Butler et al., 2004; Nagy, 2005). An average of over one-hundred scientific papers per year has been published during the past decade, mostly concerning humans, and the number of animal species studied by means of DLW is large and increasing. In his review, Nagy (Nagy, 2005) found a total of 229 species, including 95 species of birds, on which energy expenditure has been estimated with the DLW method; Ellis and Gabrielsen (Ellis and Gabrielsen, 2002) report 39 seabird species and Anderson and Jetz (Anderson and Jetz, 2005) report 86 mammalian species for which the method has been applied.

During DLW applications, a dose of heavy oxygen (^{18}O) and hydrogen (e.g. deuterium, ^{2}H) isotopes is introduced into the body of a study subject and the subsequent isotope elimination rates are measured to estimate energy expenditure (for details, see Lifson et al., 1955; Nagy, 1980; Speakman, 1997). In wild animals, the most common route of DLW administration is by injection; in seabirds, the isotopes are injected either intramuscularly (e.g. Fyhn et al., 2001; Kitaysky et al., 2000) or intraperitoneally (e.g. Humphreys et al., 2006). The labelled water equilibrates rapidly with the body water, in medium-sized birds usually within 1 h of injection (Degen et al., 1981).

Within the overall DLW framework, many different approaches with differing treatment protocols can be applied. The most commonly used protocol, the so called two-sample (TS) DLW method (Lifson and McClintock, 1966; Nagy, 1980; Speakman, 1997), requires that the animal is confined for a period of time after injection to allow for complete equilibration of isotopes with the body water pool. An initial blood sample is then taken to estimate initial isotope enrichment and the animal released to range freely. Upon recapture, a second blood sample is obtained to estimate final isotope concentrations. An alternative treatment, the single-sample (SS) DLW method (Speakman, 1997; Webster and Weathers, 1989), is applied much less frequently. This approach is less invasive than the TS DLW protocol, as it avoids the initial period of restraint and the withdrawal of the first blood sample (Webster and Weathers, 1989). However, because the initial blood samples are not obtained the initial isotope concentrations have to be estimated indirectly, commonly from a second group of animals from which an initial sample is taken according to the TS protocol. The indirect estimation of initial isotopic enrichment usually results in a slight decrease in the precision of the derived rates of energy expenditure in comparison with TS data and indirect calorimetric methods (Webster and Weathers, 1989).

DLW applications are generally believed to have only minor or no adverse effects on study animals, an assumption that is essential if energy expenditure estimates are to be meaningful (reviewed in Speakman, 1997). However, this assumption is based on limited evidence often collected incidentally to the main purpose of using the method. There are a few systematic evaluations of this assumption in free-living animals. Validation studies on laboratory animals have been mostly concerned with comparing estimates of energy expenditure with simultaneous measurements by volumetric methods (e.g. Visser and Schekkerman, 1999), and have rarely considered behavioural effects (but see Randolph, 1980; Speakman et al., 1991). Similarly, in most field studies, as has been pointed out earlier (Speakman, 1997; Uttley et al., 1994), altered behaviour of study animals is routinely not controlled for (e.g. Montevecchi et al., 1992; Weathers et al., 2002). Also, many studies that do assess potential behavioural impacts suffer from small sample sizes and consequently from low statistical power to detect effects (e.g. Williams, 1987; Wilson and Culik, 1995; Zurowski and Brigham, 1994), or measure parameters that are likely to be relatively insensitive indicators of potential effects (e.g. Sanz and Tinbergen, 1999; Weathers and Sullivan, 1993). Other studies have recorded the behaviour of treated individuals in a more opportunistic fashion to assess potential effects (e.g. Gabrielsen et al., 1987; Obst et al., 1987; Pärt et al., 1992). To our knowledge, only two systematic assessments of short-term behavioural effects of the DLW method in wild animals are available from the peer-reviewed literature (Uttley et al., 1994; Zurowski and Brigham, 1994), and one study that examined long-term mortality effects (Entwistle et al., 1994). In general, the results of these studies are equivocal. While it is mostly concluded that treatment effects are negligible (reviewed in Butler et al., 2004; Speakman, 1997), for some species reduced activity and altered foraging behaviour subsequent to DLW treatment have been reported (e.g. Jodice et al., 2003; Nilsson, 2002). Wilson and Culik (Wilson and Culik, 1995) also evaluated the impact of DLW treatment, but the reported altered behaviour was probably caused by large injection doses, a problem that today can be eliminated by the use of highly enriched DLW (*sensu* Speakman, 1997). Furthermore, despite the fact that some opportunistic observations indicate that potential adverse effects may be reduced in SS treatments (e.g. Amat et al., 2000; Cresswell et al., 2004; Webster and Weathers, 1989), no study has yet systematically compared effects of the two different DLW protocols. Critically, no study has made comparisons of animals under differing DLW protocols with untreated animals to assess which animals had the more natural patterns of behaviour. Because little evidence for altered rates of energy expenditure as a consequence of treatment effects on behaviour has been reported (e.g. Speakman et al., 1991) (but see Nilsson, 2002), the DLW method is generally assumed to provide unbiased estimates reflecting natural rates of energy expenditure (e.g. Birt-Friesen et al., 1989; Fyhn et al., 2001; Jodice et al., 2003). However, the general lack of studies of treatment effects has repeatedly provoked calls for thorough assessment of the impact of the DLW method (e.g. Entwistle et al., 1994; Speakman, 1997; Speakman et al., 1991; Zurowski and Brigham, 1994).

The aim of this study was to determine the impact of the SS and TS DLW treatment on the behaviour of black-legged kittiwakes (*Rissa tridactyla* L.; hereafter called 'kittiwakes') during the chick-rearing period. The behaviour of individuals subjected to the SS and the TS treatment was systematically compared with that of a control group of unmanipulated animals. Firstly, we examined whether kittiwakes treated with the SS and TS protocol differed in their immediate response to handling procedures by comparing their motivation to return to their nest site after release subsequent to their initial treatment. Secondly, we tested whether the nest attendance of SS and TS kittiwakes differed during the measurement periods from those of control birds. Finally, to evaluate the potential effect of modified behaviour on energy expenditure, we examined the relationship between field metabolic rate (FMR) and the amount of time birds spent away from the nest, and compared estimated rates of energy expenditure of kittiwakes derived with the SS and the TS DLW protocols.

MATERIALS AND METHODS

Experimental procedures

The study was conducted in a colony of kittiwakes on Blomstrandhalvøya (78°54′N, 12°13′E), situated *ca.* 10 km northeast of the research station Ny Ålesund in Kongsfjorden, Svalbard, Norway. We studied both partners of 33 kittiwake breeding pairs from 7th July to 13th August 2006; and both partners from 30 nests from 23rd July to 10th August 2007. The kittiwake is a medium-sized (body mass ~380 g), cliff-breeding gull species which breeds in large colonies throughout the northern Atlantic and Pacific. Males and females share chick provisioning duties and usually raise one or two chicks on Svalbard (e.g. Fyhn et al., 2001). Kittiwakes are slightly sexually size dimorphic with males weighing on average 12% more than females (e.g. Angelier et al., 2007).

Kittiwakes were randomly assigned to the SS DLW treatment group (N_{2006}=22, N_{2007}=20 individuals), TS DLW treatment group (N_{2006}=24, N_{2007}=20) or control group (N_{2006}=20, N_{2007}=20). Birds did not enter the experiment before chicks were between 15 and 21 days old (mean ± s.e.m. age: 18.07±0.03 days) to avoid potential confounding effects due to variation in behaviour and energy expenditure of birds at different stages of the breeding cycle (Fyhn et al., 2001). The two partners of a nest received the same treatment within 3 days. SS and TS kittiwakes were caught on their nests and body mass was measured using a Pesola spring balance (±5 g). If birds had not been captured previously (see below), they were individually marked with a numbered steel band and a coloured plastic band engraved with a unique 3-digit code. For easy identification they were additionally marked with red or blue marker pens on head and breast feathers. Then, each bird was injected with 0.41 ml and 0.34 ml of mixed DLW in 2006 and 2007, respectively, into the pectoral muscle using a gas-tight syringe (Hamilton Microliter Syringe, Bonaduz, GR, Switzerland; 0.5 ml). The injectate contained 41.1 and 33.9 atom percent excess (APE) deuterium (^{2}H) in 2006, and 62.1 and 56.8 APE oxygen-18 (^{18}O) in 2007. Kittiwakes in the SS treatment were released immediately after injection. Kittiwakes in the TS treatment were kept in a cotton bag for ~1 h (mean ± s.e.m. time: 64±1 min) to allow for complete equilibration of the isotopes with the body water (Lifson and McClintock, 1966; Speakman, 1997). An initial blood sample was obtained in this group by puncture of a brachial vein and collection of blood in four 70 μl heparinized microcapillary tubes. The tubes were flame sealed immediately with a butane torch. We attempted to recapture all individuals twice between 24 and 72 h after release (mean ± s.e.m. time of recapture 1: 31.5±1.6 h; recapture 2: 60.4±1.1 h). Upon all recaptures, birds were re-weighed and a blood sample was obtained as described above. An additional droplet of blood was obtained from each individual for subsequent molecular sexing following standard techniques as described in Fridolfsson and Ellegren (Fridolfsson and Ellegren, 1999). In both 2006 and 2007, two TS kittiwakes could not be recaptured at all due to prolonged absence from their nest. An additional three and four kittiwakes evaded recapture 2 in 2006 and 2007, respectively.

Behavioural observations

To gauge the immediate effect of the DLW procedure on the behaviour of an individual, we recorded the exact time elapsed between its release after treatment (SS: injection, TS: injection and 1 h restraint and blood sample) and its return to the nest (return time ±1 min). We observed nests continuously after birds were released, but terminated observations when birds had not returned to their nest after 10 h (2006) and 8 h (2007). To assess the persistence of potential effects, similar observations were performed after each of the subsequent recaptures. After recapture, nests were observed for on average 7.9±0.3 h (mean ± s.e.m.).

To determine changes in behaviour of individuals subjected to the DLW treatments in comparison to unmanipulated control birds, we monitored the nest attendance of kittiwakes during four periods: (1) pre-injection (PreInj), i.e. within about 24 h prior to injection, (2) post-injection (PostInj), i.e. within the time period between injection and recapture 1, (3) post-recapture 1 (PostRe1), i.e. within the time period between recapture 1 and recapture 2, and (4) post-recapture 2 (PostRe2), i.e. within about 24 h following recapture 2. The presence or absence of birds was recorded every 20 min for at least 10 h and 8 h within each observation period in 2006 and 2007, respectively. To facilitate immediate visual identification of all individuals, at least one partner of each nest was caught several days prior to the experiment and coloured with a permanent marker pen on the head and breast. Individuals of control nests were marked in the same way, but were not recaptured again in the course of the study.

Field metabolic rates

Isotope enrichment of blood samples was determined by isotope mass spectrometry as described in detail in Speakman and Król (Speakman and Król, 2005). Briefly, blood samples were vacuum distilled into glass Pasteur pipettes (Nagy, 1983). ^{2}H enrichment was determined using small sample pyrolysis (Król et al., 2007). ^{18}O enrichment was determined through equilibration with CO_2 of known isotopic enrichment and analysis of the gas following the small sample equilibration technique (Speakman et al., 1990). $^2H{:}^1H$ and $^{18}O{:}^{16}O$ ratios were determined by a gas source isotope ratio mass spectrometer with isotopically characterized gases of H_2 and CO_2 in the reference channels. Enrichment of the injectate was established by a dilution series with tap water and mass spectrometric analysis of five subsamples of each solution (Speakman, 1997). Four subsamples of each blood sample were analysed for isotope enrichment, and their mean value was used for all subsequent calculations. All isotope concentrations were corrected for mean natural background levels of the labels, derived from blood samples of six parent kittiwakes additionally captured during the study period of each year [method C of Speakman and Racey (Speakman and Racey, 1987)].

For TS birds, total body water at initial capture was determined from the ^{18}O dilution space (Speakman, 1997). Final body water content was calculated based on body mass and assuming a constant fraction of body water throughout the experiment. For SS birds, from which no initial blood samples were obtained, we estimated initial isotope enrichment based on the relationship of initial isotope enrichment and body mass established for TS kittiwakes each year. Then, isotope enrichment levels of all kittiwakes were converted to rates of CO_2 production (ml $CO_2\,g^{-1}\,h^{-1}$) using a single-pool model as recommended for birds with body mass <1000 g (Speakman, 1993). We assumed a fixed level of evaporative water loss of 25% [see equation 7-17 in Speakman (Speakman, 1997)] which minimizes the error in studies of birds (van Trigt et al., 2002; Visser and Schekkerman, 1999). CO_2 production rates were then converted to FMRs (kJ day^{-1}) using calorific equivalents, which were calculated based on the assumption that energy expended during the DLW measurement period was mainly derived from ingested food and that the diet fed to chicks reflected the adult diet. Regurgitation samples (N_{2006}=68, N_{2007}=132) collected opportunistically throughout the study indicated that the kittiwake diet in 2006 consisted of fish (70% of total wet mass), mainly polar cod *Boreogadus saida*, and a relatively high fraction of invertebrates (17% total wet mass crustaceans, 12% total wet mass polychaetes). In 2007, fish (99% total wet mass), mainly capelin *Mallotus villosus*, was almost exclusively present in the diet. From dried diet samples, lipid and nitrogen contents were analysed with the Soxtec and the Kjeldahl nitrogen extraction methods (Horwitz, 1975), respectively, at TosLab AS, Tromsø, Norway. Nitrogen content was converted to protein content by multiplication by a factor of 6.25 (Kleiber, 1975). The caloric equivalents, which differed only slightly between seasons (2006: 27.60 J ml^{-1} CO_2, 2007: 27.66 J ml^{-1} CO_2), were estimated according to Gessaman and Nagy (Gessaman and Nagy, 1988), assuming that only lipids and protein contributed to the energy content of the diet. For each individual that was recaptured twice after injection, FMR was calculated for two measurement periods: period 1 (FMR_{P1}) comprising the time between injection and recapture 1, and period 2 (FMR_{P2}) between recaptures 1 and 2.

Data analysis

Data on return times were right-censored and were therefore analysed using time-event analysis. Specifically, a Cox proportional hazard model (Cox, 1972) was used to test for differences in return times between the SS and the TS DLW treatment and observation period (PostInj, PostRe1 and PostRe2). In addition, we included 'year' as a factor to control for inter-annual variation. To account for repeated measurements of individuals and non-independence of partners of a pair, we entered 'individual' nested within 'nest identity' as a random component in the model.

We used a linear mixed effects (LME) model to assess differences in nest attendance between treatments (SS, TS and control), year (2006 and 2007), observation period (PreInj, PostInj, PostRe1 and PostRe2) and sexes. Nest attendance data (% of time present at nest) were arcsine transformed prior to analysis, and 'individual' within 'nest' was included as a random component. Similarly, a LME model was fitted to determine the effect of DLW treatment (SS *vs* TS) on estimated FMR. In this model, measurement period (period 1 *vs* period 2), year and sex were additionally entered as fixed factors. In both cases, full models containing all predictor variables and interaction terms were simplified based on likelihood ratio tests (LRT). Terms were eliminated from the model if their removal did not result in a significant increase in deviance. The significance of terms in the most parsimonious LME models was assessed by *F*-tests.

Finally, we examined differences between the SS and TS treatment in the relationship between estimated FMR and the amount of time birds spent off the nest to assess whether altered behaviour at sea explained variation in estimates of energy expenditure. Separate models were fitted for FMR_{P1} and FMR_{P2}. Time off nest was taken as the inverse of estimated nest attendance. To determine the effect of body mass on our results, we fitted similar models to those described above with mass-independent FMR (miFMR) as the response variable. miFMRs were calculated as the residuals of the regression of $\log_e$ FMR on $\log_e$ body mass. Furthermore, no correlation was found between FMR and deviations from the 24 h standard measurement interval (R^2=0.01, $F_{1,137}$=0.72, P=0.399), and, accordingly, FMRs entered in the models remained unadjusted. The absence of a diurnal rhythm in kittiwakes has been demonstrated previously (e.g. Bryant and Furness, 1995; Falk and Møller, 1997) and may be expected under continuous daylight in the high Arctic.

All data are reported as means ± s.e.m., except for return times, which are reported as median ± median absolute deviation due to censored measurements. Differences were considered to be statistically significant at *P*-values <0.05. All analyses were performed using the statistical software R packages nlme and kinship (R Development Core Team, 2008).

Table 1. Cox proportional hazard model to test the effect of doubly labelled water (DLW) treatment (SS or TS), observation period and year (2006 and 2007) on the return time of kittiwakes

Variable	d.f.	χ^2	*P*-value
Treatment	1	7.46	**0.006**
Period	2	1.50	0.472
Year	1	6.33	**0.012**
Treatment × period	2	6.23	**0.044**
Period × year	2	9.40	**0.009**

SS, single-sample; TS, two-sample. Observation period was post-injection, post-recapture 1 and post-recapture 2. Return time is the time elapsed between release after handling and first reappearance at the nest.

'Individual' nested within 'nest identity' was included as a random component in the model. The best model was selected by likelihood ratio tests starting from a full model including all interaction terms.

Values in bold are significant.

Table 2. Return time of kittiwakes treated with the SS and TS DLW method

		PostInj	*N*	PostRe1	*N*	PostRe2	*N*
2006	SS	9±13	22	17±24	22	25±37	21
	TS	239±301	22	111±165	21	124±148	19
2007	SS	15±15	20	6±9	20	5±7	20
	TS	215±294	20	15±21	19	8±12	15
Both	SS	11±16	42	15±20	42	10±15	41
	TS	239±320	42	38±65	40	40±59	34

Return time is the time elapsed between release after handling and first reappearance at the nest, given as median (min) ± median absolute deviation.

Data are reported for three capture events (PostInj, post-injection; PostRe1, post-recapture 1; PostRe2, post-recapture 2) in two study years (2006 and 2007) and averaged over both years.

Sample sizes (*N*) are listed for each treatment and period.

RESULTS

Effect on kittiwake behaviour

Return times

Following release, TS kittiwakes returned significantly later to their nests than SS kittiwakes (SS: 48±10 min, TS: 198±25 min, pooled for all periods and years; Table 1). This difference was most pronounced after initial capture and DLW treatment procedures, when it took on average more than 20 times longer for TS kittiwakes to resume nest attendance compared with SS birds (Table 2 and Fig. 1). However, the treatment effect persisted to a lesser degree after subsequent recaptures (significant interaction treatment × period, Table 1; Fig. 1), even though handling procedures did not differ between SS and TS kittiwakes on these recaptures. In addition, the response of kittiwakes was year dependent, with birds returning significantly faster to their nests in 2007 compared with 2006 (2006: 174±24 min, 2007: 61±11 min, pooled for all periods and treatments; Table 1). Finally, as indicated by a significant interaction between year and period (Table 1), absence from the nest after recapture was more persistent through the observation periods in 2006 than in 2007.

Nest attendance

There was a strong effect on nest attendance of TS kittiwakes after initial capture and handling (Fig. 2). While nest attendance of control and SS kittiwakes did not differ, that of TS kittiwakes was reduced by more than 40% relative to control birds (Tables 3 and 4). However, the relative difference between TS and control birds declined after subsequent recaptures to 26% and 16% for PostRe1 and PostRe2, respectively (significant interaction treatment × period, Tables 3 and 4). In addition, overall nest attendance differed significantly between years (Table 3), being on average about 30% higher in 2007 (49.5±1.2%) relative to 2006 (38.0±1.6%). Also, nest attendance declined over the whole study period in 2006, while it remained relatively similar throughout the experiment in 2007 (significant interaction year × period, Table 3). Finally, a significant effect of sex on nest attendance was found (Table 3), with females attending the nest for longer periods than males (females: 45.8±1.5%, males: 40.9±1.4%). Although there was no significant interaction between treatment and sex (LRT, χ^2_2=4.34, *P*=0.114), there was no apparent difference between sexes of control kittiwakes (females: 46.2±2.4%, males: 47.0±2.2%).

Effect on field metabolic rates

FMR estimated for the time period between injection and first recapture ($\mathrm{FMR}_{\mathrm{P1}}$) was significantly higher in SS compared with

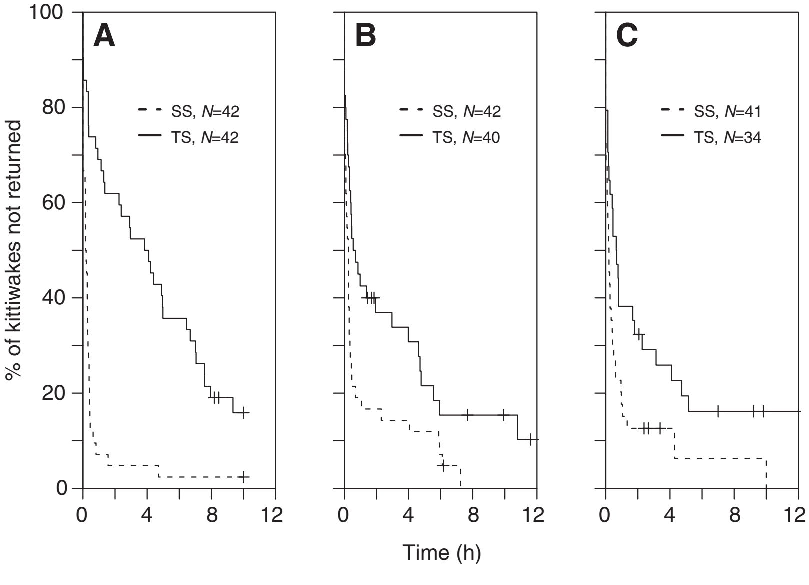

Fig. 1. Difference in return times, i.e. the time elapsed (h) between release after handling and first reappearance at the nest, of kittiwakes treated with the single-sample (SS) and two-sample (TS) doubly labelled water (DLW) method. Data are pooled over both study years and presented as the percentage of kittiwakes that had not yet returned to their nest at a particular time. Censored measurements are indicated with + symbols. (A) return time after initial capture and DLW treatment. (B) Return time after first recapture. (C) Return time after second recapture. See text for details.

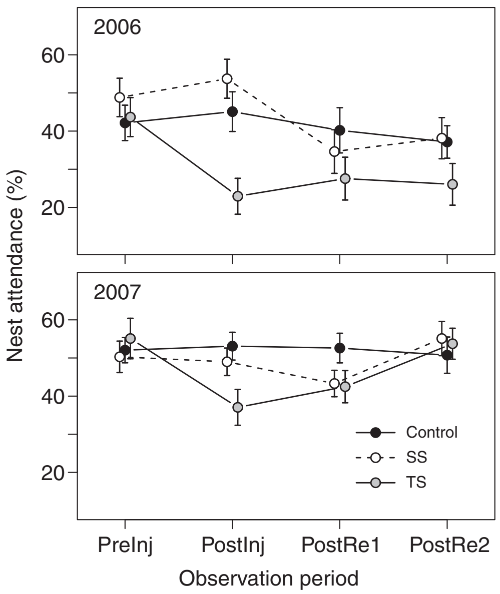

Fig. 2. Difference in nest attendance (%) between kittiwakes treated with the SS and TS DLW method and unmanipulated control birds. Nest attendance of individual kittiwakes was observed in four different periods during each study year: within 24 h prior to injection (PreInj), within the time period between injection and recapture 1 (PostInj), within the time period between recapture 1 and 2 (PostRe1), and within 24 h after recapture 2 (PostRe2). The presence or absence of individual kittiwakes was recorded every 20 min for at least 10 h and 8 h per period in 2006 and 2007, respectively.

TS birds (14.9%, Table 5). This difference was similar in the two study years (2006: 17.9%, 2007: 11.1%; interaction treatment × year: LRT, χ^2_1=0.09, P=0.760) and was present in both males and females (interaction treatment × sex: LRT, χ^2_1=0.34, P=0.535). Overall, FMR differed between the sexes, with males spending on average 18.6% more energy than females (Table 5). This difference was related to the sexual dimorphism in body mass in kittiwakes, as mass-independent FMR did not differ between males and females (LME, $F_{1,37}$=1.42, P=0.240). Also, there was a significant difference in estimates of energy expenditure between measurement periods ($\mathrm{FMR}_{\mathrm{P1}}$ *vs* $\mathrm{FMR}_{\mathrm{P2}}$, Table 5). However, this difference seemed to be driven by an unaccountably high $\mathrm{FMR}_{\mathrm{P2}}$ of males in 2006, leading to a significant 3-way interaction (period × year × sex, Table 5). The estimate of energy expenditure of this group of male kittiwakes was elevated by 32.9% in comparison with average male FMR. The reasons for these high estimates are unknown. When removed from the dataset, the effect of measurement period on FMR became non-significant (LRT, χ^2_1=0.74, P=0.391), also indicating that the difference in FMR between SS and TS treatment persisted in measurement period 2 (interaction treatment × period: LRT, χ^2_1=0.59, P=0.444).

Furthermore, we found that $\mathrm{FMR}_{\mathrm{P1}}$ was positively related to the amount of time kittiwakes spent off the nest (LME, $F_{1,33}$=6.42, P=0.016). However, this relationship was dependent on the DLW treatment (significant interaction time off nest × DLW treatment: LME, $F_{1,33}$=8.46, P=0.007). While $\mathrm{FMR}_{\mathrm{P1}}$ was strongly positively correlated with time off nest (linear regression: R^2=0.46, $F_{1,38}$=31.75, P<0.001) in SS birds, this was not the case in birds treated with the TS procedure (R^2=0.02, $F_{1,35}$=0.64, P=0.430; Fig. 3). In contrast, there was no difference between DLW treatments in the relationship between $\mathrm{FMR}_{\mathrm{P2}}$ and time off nest (LRT, χ^2_1=1.00, P=0.318).

Table 3. Linear mixed effects model to test the effect of treatment, observation period and year on the nest attendance (%) of kittiwakes

Variable	d.f.	*F*	*P*-value
Treatment	2,79	6.14	**0.003**
Year	1,79	15.62	**<0.001**
Period	3,359	3.30	**0.020**
Sex	1,79	4.51	**0.037**
Treatment × period	6,359	3.09	**0.006**
Year × period	3,359	2.82	**0.039**

Treatment was SS or TS DLW method and control; observation period was post-injection, post-recapture 1 and post-recapture 2; year was 2006 and 2007.

‘Individual’ nested within ‘nest identity’ was included as a random component in the model. The best model was selected by likelihood ratio tests starting from a full model including all interaction terms.

Values in bold are significant.

Table 4. Nest attendance (%) of kittiwakes treated with the SS or the TS DLW method and unmanipulated control birds during four observation periods

		N	PreInj	PostInj	PostRe1	PostRe2
2006	Control	20	42.2±4.6	45.1±5.2	40.2±5.9	37.2±4.3
	SS	22	48.8±5.0	53.7±5.1	34.6±5.7	38.1±5.4
	TS	24	43.6±5.1	22.9±4.7	27.5±5.6	26.0±5.5
2007	Control	20	52.1±3.3	53.1±3.6	52.6±3.9	50.7±4.8
	SS	20	50.3±4.1	49.0±3.6	43.3±3.5	55.1±4.5
	TS	20	55.1±5.3	37.1±4.7	42.5±4.2	53.7±4.1
Both	Control	40	47.1±2.9	49.1±3.2	46.4±3.6	43.8±3.3
	SS	42	49.5±3.3	51.5±3.2	38.8±3.4	46.2±3.7
	TS	44	48.9±3.7	29.3±3.5	34.1±3.8	36.7±4.3

Nest attendance is given as means ± s.e.m.

Observation period was pre-injection (PreInj), post-injection (PostInj), post-recapture 1 (PostRe1) and post-recapture 2 (PostRe2) (see text for details) in two study years (2006 and 2007) and averaged for both years.

Sample sizes (*N*) are listed for each treatment.

Table 5. Linear mixed effects model to test the effect of DLW treatment (SS or TS), observation period, year (2006 and 2007) and sex on the field metabolic rate (kJ day^{-1}) of kittiwakes

Variable	d.f.	*F*	*P*-value
DLW treatment	1,39	14.04	**0.001**
Period	1,55	13.73	**0.001**
Year	1,39	0.14	0.712
Sex	1,37	15.36	**<0.001**
Period × year	1,55	0.68	0.412
Period × sex	1,55	8.02	**0.007**
Year × sex	1,37	10.12	**0.003**
Period × year × sex	1,55	8.62	**0.005**

Observation period was post-injection, post-recapture 1 and post-recapture 2.

‘Individual’ nested within ‘nest identity’ was included as a random component in the model. The best model was selected by likelihood ratio tests starting from a full model including all interaction terms.

Values in bold are significant.

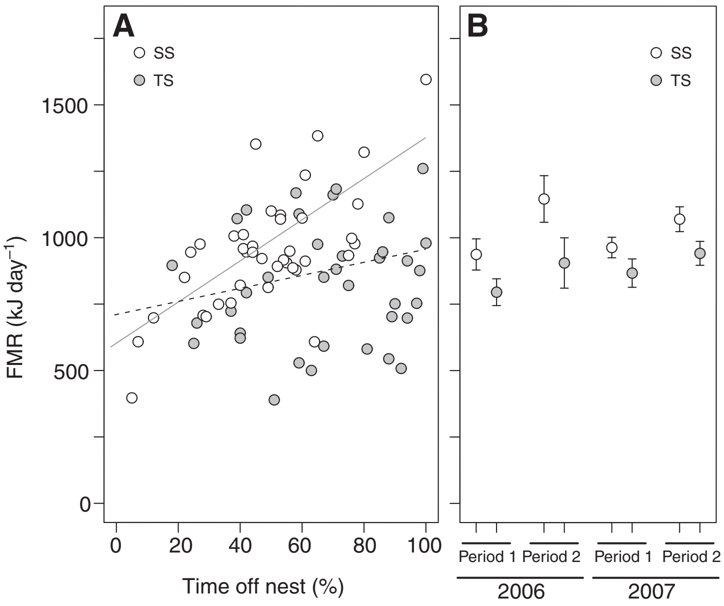

Fig. 3. (A) Relationship between field metabolic rate (FMR, kJ day^{-1}) and the amount of time spent away from the nest (%) of kittiwakes treated with the SS or TS DLW method. FMR was measured between initial release and first recapture (see text for details). While in SS kittiwakes FMR and time off nest were positively correlated (solid line; R^2=0.45, F=31.75, P<0.001), this was not the case in TS birds (broken line; R^2=0.02, F=0.64, P=0.430). (B) Difference in FMRs (means ± s.e.m.) between the treatments in period 1 (time between injection and recapture 1) and period 2 (time between recaptures 1 and 2) in two study years (2006 and 2007).

DISCUSSION

Altered behaviour

Our results show that the application of the DLW method can adversely affect the behaviour of study animals. In contrast to birds subjected to the SS DLW procedure, we found a strong behavioural response of kittiwakes to the commonly used TS DLW method, which resulted in a reduced motivation to return to their nests and a reduced overall nest attendance after initial capture and treatment (injection and captivity). Kittiwakes treated according to the TS protocol did not return to their nests for on average about 4 h, a 20-fold increase in comparison with SS birds. In addition, TS kittiwakes reduced their nest attendance between initial release and first recapture by more than 40% relative to unmanipulated kittiwakes. However, we found that the effect of the TS treatment on both behavioural parameters persisted, although it was less pronounced, after subsequent handling and bleeding procedures. Following recaptures 1 and 2, TS kittiwakes remained absent from their nests for about 40 min, more than twice as long as kittiwakes treated with the SS DLW method, and overall nest attendance remained ~20% below that of unmanipulated birds. This was the case even though treatment at recapture did not differ between SS and TS birds, suggesting that TS birds responded more strongly due to the additional stress experienced during the initial DLW treatment. Furthermore, although return times and nest attendance of TS kittiwakes were affected in both study years, their sensitivity to the DLW treatment seemed to depend to some degree on annual conditions. The TS procedure affected kittiwakes more strongly in 2006 than in 2007, as indicated by considerably increased return times after initial treatment and subsequent recaptures. Similarly, nest attendance of TS birds was more strongly and more persistently reduced in response to DLW treatment in 2006. These differences may be related to differences in prevalent foraging conditions between study years. A lower proportion of fish in the diet (see Materials and methods), overall reduced nest attendance and lower chick fledging success (Welcker et al., 2010) in 2006 compared with 2007 indicate pronounced food restrictions in the former year (Gill et al., 2002; Kitaysky et al., 2000). Thus, kittiwakes seemed to be more severely affected by the TS treatment during an unfavourable year (2006), suggesting that stress imposed by the DLW procedure was additive to other external stressors, and birds may tolerate handling stress to a larger degree when overall conditions are favourable. This may also explain contrasting results in other studies, in which no effect of the TS DLW method on return times (Humphreys et al., 2006) or nest attendance (Golet et al., 2000; Siegel et al., 1999) was found.

In contrast, we found no evidence of a negative effect of the SS DLW method. In both years, the behaviour of kittiwakes treated with the SS method did not differ from that of unmanipulated birds. SS kittiwakes returned to their nests within minutes after initial treatment and release, and resumed normal nesting behaviour as indicated by nest attendance similar to that of control birds. These results corroborate circumstantial evidence reported in previous studies (Amat et al., 2000; Cresswell et al., 2004; Obst et al., 1987; Webster and Weathers, 1989) that a potential adverse impact of the TS protocol on the behaviour of study animals may be substantially reduced, or completely avoided, when the SS method is applied. However, it should be noted that in comparison to many other seabird species, the kittiwake has generally been regarded as a species that is relatively insensitive to human disturbance and capture and handling procedures (Golet et al., 2000; Sandvik and Barrett, 2001; Thomson et al., 1998), which may partly explain why the kittiwake is among the species most intensively studied by the DLW method (e.g. Gabrielsen et al., 1987; Jodice et al., 2006; Kitaysky et al., 2000). In more sensitive species, even the minor treatment procedures of the SS protocol may elicit adverse effects. For example, Furness and Bryant (Furness and Bryant, 1996) reported an unusually long post-treatment absence from the nest in northern fulmars (*Fulmarus glacialis*) after application of the SS DLW method, and nest attendance in one of four petrel species handled according to the SS approach was affected in a study by Hodum and Weathers (Hodum and Weathers, 2003). For these species, modifications of the DLW method that aim at further minimization of handling could be advantageous [e.g. administration of DLW through injected prey items (Anava et al., 2002); collection of faecal samples (Gotaas et al., 1997); non-invasive blood sampling (Voigt et al., 2003)], when validation studies have shown their applicability in different species.

We administered the dosage of DLW by intramuscular injection into the pectoralis major. This method has been suspected to cause discomfort when large volumes of DLW are applied, leading to reduced foraging activity (Wilson and Culik, 1995). The lack of a behavioural effect in response to the SS protocol suggests that altered behaviour in TS birds was not related to the dosing procedure but rather to the period of captivity after injection, the main difference between handling procedures. However, we cannot rule out the possibility that discomfort may have been caused by the combination of intramuscular injection and subsequent restriction during which the bird's breast muscle may have tightened. Intraperitoneal injection might therefore mitigate adverse effects. The blood sampling itself, which was done initially in the TS but not the SS group, is generally regarded to have no adverse effects (e.g. Hoysak and Weatherhead, 1991). More importantly, in our study SS kittiwakes that were blood sampled at recapture returned to their nests rapidly, indicating limited impact of this part of the protocol. Even though we did not observe signs of severe stress in captive kittiwakes, and birds remained calm throughout the period of restraint, part of the observed effect may have been related to the disarrangement of feathers during captivity. Birds may then spend considerable time rearranging their plumage after release.

As indicated above, the impact of the DLW method on the behaviour of study subjects may depend on several factors. Two factors inherent in our study design may potentially have led to an aggravation of negative effects. Firstly, it has been suggested that individuals may react more sensitively to the treatment when both partners of a pair are treated (Uttley et al., 1994). Since we consistently applied the DLW method in both partners of pairs, the disturbance may have been minimized if only one partner had been manipulated. Secondly, the timing of the experiment with respect to the breeding cycle may have had a reinforcing effect. Although incubating birds are generally thought to be more susceptible to perturbations than chick-rearing birds (Obst et al., 1987; Uttley et al., 1994; Williams, 1987), the opposite has been observed in kittiwakes (Fyhn et al., 2001). Consideration of these factors and corresponding adjustment of the study design may therefore reduce the negative impact of the treatment. However, it seems unlikely that behavioural effects can be entirely avoided.

Our results are in contrast to earlier studies on the kittiwake which indicated no adverse effect of the TS DLW treatment (Golet et al., 2000; Humphreys et al., 2006; Thomson et al., 1998), but support previous peripheral evidence of altered behaviour in this species (Fyhn et al., 2001; Jodice et al., 2003) and a reduced impact when the SS protocol is applied (Jodice et al., 2003). The paucity of information on behavioural responses in most other wild animal species calls for more attention to the potential effects of DLW application.

Effects on estimated FMR

Our results demonstrate that estimated rates of energy expenditure may differ considerably when derived by the different approaches of the DLW methodology. Estimated rates of energy expenditure were about 15% lower in kittiwakes treated with the TS DLW method than in the SS treatment group. This effect of DLW treatment was present in both study years and in both sexes, and seemed to persist in both measurement periods. Our data suggest that reduced $\mathrm{FMR_{P1}}$ of TS birds was related to behavioural modifications while birds were at sea. While in SS birds there was a strong positive correlation between $\mathrm{FMR_{P1}}$ and the amount of time spent at sea, this was not the case in individuals subjected to the TS protocol. It has been demonstrated that individual variation in energy expenditure in kittiwakes is largely related to differences in the amount of time they spend actively foraging when away from the colony (Jodice et al., 2003). This indicates that foraging activity of the TS birds in our study was reduced, and birds may have been inactive for extended time periods while at sea, possibly to recover from handling stress. This interpretation is supported by the findings of Jodice and colleagues (Jodice et al., 2003), who continuously recorded the behaviour of radio-tagged kittiwakes treated with the SS and TS DLW method. They reported that TS birds dedicated nearly a third of their time-budget to recovering activities such as loafing close to their breeding colony after DLW treatment. In contrast, SS kittiwakes rarely showed this behaviour (Jodice et al., 2003). Furthermore, similar to our results, Jodice and colleagues (Jodice et al., 2003) reported a tendency towards lower estimates of energy expenditure in TS kittiwakes compared with SS-treated conspecifics. However, in their study differences between DLW treatments were statistically non-significant, possibly due to the low sample size. Reduced forging activity in birds treated with the TS method was also reported in common terns [*Sterna hirundo* (Uttley et al., 1994)] and in marsh tits [*Parus palustris* (Nilsson, 2002)]. After administration of DLW, 50% of treated marsh tits failed to resume normal feeding activity for several hours, leading to biased estimates of energy expenditure (Nilsson, 2002).

Our data suggest that the difference in FMR between SS and TS treatments persisted in the second measurement period. This was the case even though the relationship between FMR and time at sea differed between DLW treatments only during the first period subsequent to initial capture and injection. During the second measurement period (between recapture 1 and 2), FMR was positively related to time at sea in both SS and TS kittiwakes. Hence, altered behaviour at sea seemed primarily to be a consequence of the initial treatment. However, as indicated by persisting effects of the TS treatment on nest attendance and return times, birds did not entirely resume normal breeding behaviour during the latter part of the experiment, which may explain persisting differences in FMR estimates. Although these effects of the TS protocol appeared to persist beyond the immediate period following initial handling, the main impact occurred immediately after the first release. An additional way to ameliorate the impact of the TS protocol on FMR measurements would be to extend the measurement period so that the impact of an initial disturbance is minimized. For example, in this study the TS-treated birds did not return to the nest for more than 3 h following release. This period of absence equals about 14% of a 24 h recapture interval, but only about 5% of a 72 h recapture interval. Extending recapture beyond multiple periods of 24 h is known to be advantageous for the DLW method as it minimizes the impact of substantial day to day variability in FMR estimates (Speakman et al., 1994; Berteaux et al., 1996), but an additional advantage may be to minimize the impact of initial behavioural disruption. Another way to abate effects of the TS method would be to dose animals at times when they are likely to be inactive. For example, catching diurnally active animals at the end of the light period and releasing them at the onset of darkness may minimize the behavioural impact of post-release inactivity on energy expenditure measurements.

Theoretically, the observed bias in energy expenditure may be due to a systematic methodological error, possibly caused by the indirect estimation of initial isotope enrichment of SS birds. We used the relationship between initial enrichment and body mass established for TS kittiwakes each year to predict initial enrichment of SS birds. In both years, initial enrichment and body mass were closely related (2006: R^2=0.89, 2007: R^2=0.96), indicating that prediction of enrichment based on body mass did not lead to large or systematic errors. Also, validation studies have shown that, although the small additional error introduced by indirect estimation of initial enrichment may lead to slightly reduced precision of the resultant FMR estimates, the SS protocol did not lead to reduced accuracy in comparison with the TS method (Webster and Weathers, 1989). Alternatively, a bias may have been introduced if the energy expenditure of TS kittiwakes that evaded recapture due to long periods of absence from the nest site was systematically higher than that of captured birds. However, since there was no effect of time off nest on FMR in the TS group, this is unlikely to account for the lower estimated energy expenditure of the TS group.

In conclusion, our results demonstrate a negative impact of the TS DLW treatment on the subsequent behaviour of the study subjects. This effect was primarily related to initial handling procedures but persisted to a lesser degree for longer time periods, especially when birds experienced additional external stress. Behavioural modifications may have caused biased estimates of energy expenditure derived by the TS method. Our data contrast with several earlier studies which, often based on circumstantial evidence, assumed a negligible impact of the DLW procedure on the behaviour of free-living animals (e.g. Gabrielsen et al., 1987; Moreno et al., 1997; Obst and Nagy, 1992) (but see Nilsson, 2002). This assumption may be fallacious, and our study emphasizes the need to control for behavioural impacts when the DLW method is applied. Further research is warranted that systematically compares the behaviour of experimental and unmanipulated individuals to evaluate potential effects for a larger range of species. Although the lack of apparent negative effects in birds subjected to the SS procedure suggests that this method may result in unbiased estimates of energy expenditure, its precision critically depends on a precise estimation of initial enrichment. In our study, the predicted initial enrichment of SS birds was based on a large sample of TS animals and was hence fairly robust. However, this approach may often not be feasible. The choice of sampling protocol may therefore depend on the trade-off between the need for both accuracy and precision, and knowledge about the impact of the TS protocol on the study species. When the TS method is applied, adjustments of the sampling protocol such as prolongation of the measurement interval or the timing of the treatment with respect to diurnal activity rhythms may help to mitigate potential negative effects.

LIST OF ABBREVIATIONS

APE	atom percent excess
DLW	doubly labelled water
FMR	field metabolic rate (kJ day^{-1})
FMR$_{P1}$	FMR between injection and recapture 1
FMR$_{P2}$	FMR between recaptures 1 and 2
^{2}H	deuterium
LME	linear mixed effects (model)
LRT	likelihood ratio test
miFMR	mass-independent FMR
^{18}O	oxygen-18
SS	single-sample (DLW treatment)
PostInj	post-injection
PostRe1	post-recapture 1
PostRe2	post-recapture 2
PreInj	pre-injection
TS	two-sample (DLW treatment)

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