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Effects of the Doubly Labelled Water Procedure on Great Tits *Parus major* feeding young

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Short title: Effects of doubly labelled water

Capsule Female Great Tit *Parus major* feeding rate behaviour was highly variable between individuals, but no effect of the doubly labelled water (DLW) procedure on female visit rate was detected, whereas visit rates by males, which were neither trapped or manipulated, increased following trapping and manipulation of their partners.

Aims To test the assumption that the subject's behaviour is normal during the measurement period when using DLW to measure energy expenditure.

Methods Visits to the nest box by parents feeding young were counted separately for females and males on the day before and the day after the female only was trapped to measure her energy expenditure using DLW. Visit rates were also counted for control pairs.

Results Female visit rates did not differ before and after manipulation, or between experimental and control pairs, but bird behaviour was highly variable between individuals. In contrast, the visit rates of both experimental and control males, which were not trapped, increased on the second day.

Conclusion The results for female behaviour supported the assumption of normality, but a small subset of particular individuals may be prone to adverse reactions. The response of the males may have been a reaction to disturbance at the nest.

The measurement of energy expenditure by free-living animals was revolutionised by the introduction of the doubly labelled water (DLW) method (Lifson & McClintock 1966, Speakman 1998, Butler *et al*. 2004). This technique uses the differential elimination from the body of isotopically labelled oxygen and hydrogen to provide a measure of carbon dioxide production and hence an estimate of energy expenditure (Speakman 1997). Elimination rates of the two isotopes are calculated from the difference between the initial enrichment, following injection (or ingestion) and equilibration with the body water, and the final enrichment after a set time period during which the subject is free to go about its normal activities. Isotopic enrichments are usually determined from blood samples. In most studies of free-living animals, the period during which energy expenditure is measured is usually set at 24 hours, or multiples thereof, to coincide with diurnal rhythms of activity (Speakman & Racey 1988).

The protocol required to initiate a two-sample DLW measurement, i.e. capture, handling, injection, restraint during equilibration (generally up to 1 hour birds) and blood sampling, must, in addition to the potential for physical discomfort or damage, cause a degree of stress to the subject, but how long any adverse effects persist is largely unknown (Speakman *et al*. 1991). In free-living birds, it is generally assumed that the subject's behaviour during the measurement period between initial capture and resampling is normal. There have been relatively few attempts to test this assumption in the field and most studies have found little or no effect (e.g. Obst & Nagy 1992, Uttley *et al.* 1994, Zurowski & Brigham 1994, Wilson & Culik 1995, review in Speakman 1997). However, in a recent comprehensive study of Black-legged Kittiwakes *Rissa tridactyla* (Schultner *et al.* 2010), the time taken to return to the nest increased (20 times) and nest attendance decreased (40%) in experimental birds compared to controls following the initial procedures of the two-sample method (i.e. capture, injection, equilibration period, blood sampling, release). Changes in return times and nest attendance were also noted in Northern

Fulmars *Fulmarus glacialis* labelled with DLW, but there was no correlation between measured energy expenditure and length of absence (Furness & Bryant 1996). In contrast, energy expenditure in the kittiwakes was reduced (15%) compared to birds treated with a single-sample procedure (capture, injection, release, i.e. no initial blood sample and hence no need for an equilibration period). These results are consider in more detail in the discussion.

Some studies in small passerines have also recorded changes in behaviour. Ward (1996) found that the technique increased the probability of desertion in laying Barn Swallows *Hirundo rustica*. In studies of breeding Great Tits *Parus major* (Tinbergen & Dietz 1994, Tinbergen & Verhulst 2000), between a quarter and a third of females caught at night on young in their nestboxes for injection and initial sampling, did not roost in the box on the second night and had to be retrapped in the morning when feeding the young. A more problematic change in behaviour was reported by Nilsson (2002) who found that six out of 12 female Marsh Tits *Poecile palustris* trapped and manipulated during the day did not resume feeding young for 4-10 h (mean = 5.7 h) after release which reduced their energy expenditure by 46%, on average, compared to the six females which resumed feeding within an hour. In a study of Pied Flycatchers *Ficedula hypoleuca* two out of 14 females and one out of 11 males were not seen to feed the young during one hour of observation during the measurement period (Moreno *et al*. 1995). However, this contrasts with results for Collared Flycatchers *Ficedula albicollis* where feeding rates did not differ between intraperitoneally injected and uninjected females (Pärt *et al*. 1992).

During measurements of energy expenditure using DLW in free-living female Great Tits feeding young in 2003 (Hinsley *et al.* 2008, Hinsley *et al.*, unpubl. data), female behaviour in response to trapping was highly variable. Most individuals were relatively easy to both trap and retrap, but some could not be retrapped and a few could not be caught at all. This observation raised questions about the behaviour of manipulated females during the measurement period. In

this paper, we investigated nestling feeding behaviour, recorded as the visit rate to the box, of both male and female Great Tits before and after the female only was trapped for injection with DLW and initial sampling. For comparison, we also observed pairs in which females were not trapped.

METHODS

Observation and the DLW procedure

Observations (by PR and LF, *c.* 50% each) were made in two mixed deciduous woodlands (157 ha and 132 ha) in Cambridgeshire, eastern England, during May and June of 2004. We watched Great Tit pairs feeding young (*c.* 10 days old) in nestboxes using a telescope from distances of 40 to 80 m depending on the density of cover around the box. For each pair, we made observations at the same time of day (either between *c.* 0600 and 0900 hours or *c.* 1500 and 1800 hours) on two, usually consecutive, days (Tables 1). During each observation period, we watched the birds for between 1.7 and 2.5 hours and counted the number of visits to the box made by each of the male and the female. We sexed the birds using plumage characteristics, in particular, the width of the black belly stripe between the legs (wider and darker in males) and the colour of the crown (black and glossy in males, dull black and/or greyish in females) and the generally brighter appearance of the male. Visits where the sex of the bird could not be distinguished were recorded separately. To maintain concentration, we counted visits continuously for a set time (usually 25 minutes) and then took a short break (usually five minutes), but without leaving the observation point. Thus an observer was present for longer than the time during which visits were actually counted, the maximum time per observation period being about 3.0 hours. Wherever possible, we attempted to keep disturbance to the birds at a minimum by approaching the observation point without walking near the nestbox and having the observer covered by camouflage material. Some

additional, but minor, disturbance was caused when establishing the observation points and clearing sight lines (usually one to three days before starting observations).

Experimental pairs were those where the female was trapped as part of the procedure to measure her energy expenditure using doubly labelled water. We recorded the visit rates of 11 such experimental pairs; the first observation period being on the day before the female was first trapped (see below) and the second being within the 24 hours after trapping and before the attempt to retrap the female. We also recorded visit rates, usually on consecutive days (Tables 1), for six control pairs in which neither sex was trapped. However, the young of two control pairs (pairs 2 and 6) were ringed and weighed between the two observation periods, and those of another pair (pair 5) were ringed and weighed two days before the first observation period. Observations of an additional three pairs (one experimental and two control) were omitted because the sexes of the birds could not be reliably distinguished and the nests of two further putative control pairs were predated prior to observation. This resulted in a smaller than planned control group, but by observing experimental females before and after manipulation they did, in effect, also act as their own controls. Most experimental females were more easily identified during the second observation period due to the placement of a white mark on the tail (see below). Experimental and control pairs were assigned at random.

We trapped experimental females in the box using a manually-operated trap door built into the box. Once caught, the female was ringed (or her ring number recorded), aged using plumage characteristics (Svensson 1992) and sex was confirmed by the presence of a brood patch. We measured the length (maximum chord, Svensson 1992) of one wing and weighed the bird to 0.01g (0.06% of body mass) using a portable top pan balance (ACB 300, Adam Equipment Co. Ltd.). We also temporarily marked the tip of her tail with white correction fluid (Tipp-Ex, BIC Deutschland GmbH & Co.) to increase the ease of distinguishing her from the male when

attempting to trap her for the second time 24 hours later. After processing, we injected the bird

intraperitoneally with 0.08 ml of doubly labelled water and placed her in a cloth bag for 30

minutes (Thomas *et al.* 2001) to allow the injectate to equilibrate with the body water. We then

collected a blood sample (maximum volume 75 μl) from the brachial vein in one wing and

returned her to the nestbox. The procedures, from trapping to release back into the box, took a

maximum of about one hour. Procedures were carried out at a location away from the nestbox to

allow the male to continue to feed the young. For 10 out of 11 of the experimental pairs, we

ringed and weighed the young during the 30 minute equilibration time. Thus all males were

disturbed by the trapping and return of the female and most were also disturbed by the ringing of

the young which was done at the box and took a maximum of 15 minutes. At the one box (pair 4,

Tables 1) where the young were not ringed on the day the female was trapped for the first time,

we caught the male by mistake and kept him in a bag for about 30 minutes until the female was

caught, whereupon he was released into the box. None of the observed or trapped birds deserted

(but see discussion) and none of the injected females showed any apparent adverse physical

response to the injection. All broods fledged without loss after manipulation and/or observation.

Statistical analyses

The aim of the work was to test for an effect of the DLW methodology on bird behaviour,

measured as visit rate to the box, by comparing across treatments (experimental versus control

pairs) and also across days (Day 1 versus Day 2) within pairs. In addition, given that only females

were manipulated, we also investigated the effect of sex on these responses. The data had a

hierarchical structure, i.e. pairs within treatments and birds within pairs, with days as a repeated

measures factor within birds. We tested for effects of treatment, sex, days and their interactions at

three levels in the hierarchy using nested analysis of variance (ANOVA) as follows: i) treatment

effects - between pairs, ii) sex effects – between birds within pairs, iii) day effects – between days within birds. We found a strong effect of sex and thus also examined the responses of males and females separately using nested ANOVA.

   Foraging behaviour and hence visit rates may also have been influenced by brood size (e.g. Nilsson 2002) and timing within the breeding season (e.g. Thomas et al. 2001). In addition, if behaviour were disturbed by the DLW procedures, then visit rates of experimental birds may have been affected by the length of time elapsed between manipulating the female and the start of the second set of observations. Within treatments, there were no correlations between visit rates and brood size on either Day 1 or Day 2. Between treatments, brood size was slightly larger for control pairs, but the difference was small (control: mean $\pm$ sd = 9.2 $\pm$ 0.8, n = 6; exptl: mean $\pm$ sd = 8.3 $\pm$ 1.4, n = 11; two-sample t-test: t_{14} = 1.75, P = 0.101) and was contrary to expectation in relation to visit rates, i.e. visit rates were greater for experimental males than control males and did not differ significantly between treatments for females (Tables 2-4). Therefore, we did not correct visit rates for brood size in the analyses. Effects on visit rates of time elapsed between manipulation and observations and of timing within the breeding season were examined using Pearson's correlation. Female body mass at first and second trapping and the brood size and mean body mass of young in experimental and control broods were compared using paired and two-sample t-tests as indicated. All ANOVAs were carried out using Genstat Release 9 and all other comparisons using Minitab Release 12.

RESULTS

Visit rates varied substantially, between and within pairs, for both the experimental and the control birds (Table 1). Mean visit rates for experimental and control pairs split by sex and day are shown in Table 2. Preliminary analysis (two-sample t-test) indicated that visit rates were

higher for experimental males on both Day 1 and Day 2, but that there were no differences for females in either treatment (Table 2). In the overall nested ANOVA, at the highest level of comparison (between pairs), there was no overall effect of treatment on visit rates (Table 3). This was probably due, at least in part, to the mean differences in visit rates between experimental and control pairs being positive for males, but negative for females (Table 2). At the next level (between birds within pairs), there was strong evidence of an effect of sex on visit rates and of an interaction between sex and treatment. Thus visit rates differed between the sexes (more visits by males) and this difference was affected by treatment (more visits by experimental males). At the final level (between days within birds), there was a strong effect of day on visit rates and of an interaction between day and sex. Thus visit rates differed between days and this difference was again affected by sex (more visits by males on Day 2). However, there was no additional effect of treatment (day x sex x treatment interaction)

Examining the sexes separately highlighted the differences described above. Contrary to expectation, the visit rates of females, the manipulated sex, showed no effect of treatment and did not differ between Days 1 and 2 (Table 4a). For males, visit rates differed between treatments (although the effect was only just significant) and also differed (highly significantly) between Days 1 and 2 (Table 4b). Thus experimental males made more visits than control males, and all males made more visits to the box on Day 2, despite no significant change in female visit rates (Table 2). However, as with the overall nested ANOVA, evidence for an effect of treatment on the difference in visit rates by males between days was not significant (day x treatment interaction, Table 4b). Assuming no substantial increase in chick food demand on Day 2, this suggested that all males may have been responding to the presence of the observer.

However, this did not explain why experimental males had higher visit rates than control males. An increase in male visit rates on Day 2 for the experimental birds might have been

expected if those of the females had decreased, but this was not the case (Table 2). Also, there was no correlation between the change in male and female visit rates from Day 1 to Day 2 ($r = 0.37$, $n = 11$, $P = 0.27$) and the trend in the correlation between male and female visit rates on Day 2 was positive ($r = 0.58$, $n = 11$, $P = 0.059$) rather than negative. There was no difference in the degree of disturbance at nestboxes (measured as the number of routine nest recording visits) used by experimental and control pairs prior to observations on Day 1 (exptl.: mean no. visits $\pm$ sd = 4.9 ± 1.0, $n = 11$; control: mean = 4.8 ± 0.8, $n = 6$). Most experimental pairs were observed in the afternoon whereas most control pairs were observed in the morning, but time of day, i.e. morning/afternoon, had no effect on male visit rates ($F_{1,15} = 0.31$, $P = 0.58$). Thus the difference may have been due to chance and/or the relatively small sample sizes, especially of control pairs.

For experimental females, there was no correlation between the length of time elapsed between trapping (measured from the time at which the female was returned to the box) and the start of the post-trapping observations on Day 2, and the change in the visit rate from Day 1 to Day 2 ($r = 0.33$, $n = 11$, $P = 0.32$), i.e. there was no evidence of an effect of a "recovery period" on the change in female visit rate. For experimental males, there was a positive correlation between elapsed time and the change in visit rate ($r = 0.70$, $n = 11$, $P = 0.016$). This correlation was heavily influenced by the two longest intervals without which the significance of the correlation was reduced ($r = 0.68$, $n = 9$, $P = 0.043$). For both experimental and control birds, there was no correlation between the timing of the observations within the breeding season (measured using the date of the first observation period, i.e. Day 1) and visit rates during either observation period. There was also no correlation between the daily energy expenditure of the experimental females (Hinsley *et al.* unpbl. data) and female or male visits rates on either Day 1 or Day 2 or between the changes in the rates between the two days.

There was no significant difference in the body mass of experimental females at the beginning (mean mass ± sd = 18.07 ± 0.56 g, $n = 11$) and the end (mean mass = 17.96 ± 0.58 g) of the DLW measurement period, (paired t-test: $t_8 = 1.54$, $n = 9$, $P = 0.16$) and the mean change in mass was small: mean change = -0.12 ± 0.23 g, mean % change = - 0.64 ± 1.25, $n = 9$. The mean body mass of young in experimental and control broods did not differ significantly (exptl: mean chick mass ± sd = 17.6 ± 0.55 g, $n = 11$; control: mean = 18.3 ± 0.93 g, $n = 6$; two-sample t-test: $t_6 = 1.54$, $P = 0.174$).

DISCUSSION

Visit rates were highly variable, both within and between pairs for both experimental and control birds (Table 1). Similar variation has also been recorded in other studies of tits (e.g. Sanz *et al*. 1998, Tinbergen & Verhulst 2000, Nilsson 2002). There was no significant difference in visit rates of experimental females before and after trapping, and females that were not seen to visit the box on Day 2 tended to be those with low Day 1 visit rates. However, two out of the three females not observed on Day 2, were successfully retrapped later as they fed the young. Of these two, one (pair 2) was relatively easy to catch and one (pair 1) was difficult. During the Day 1 observations, i.e. prior to trapping, this latter female was twice seen to be deterred from entering the box, apparently by the nearby presence of a Common Blackbird (*Turdus merula*) and on five other occasions was seen to approach with food, only to leave again without entering the box. This was all suggestive of a naturally nervous bird (Verbeek *et al.* 1999, Drent *et al.* 2003). Similarly, the third female (pair 4) not seen on Day 2 could not be retrapped; although present, she would not go to the box in the presence of the operator. This female had bred in the same box in 2003, and had been difficult to retrap. The other female (pair 7) which was not retrapped was "caught" in the box twice, but was able to escape. Thus the birds for which measurements can be

obtained may not be a truly random sample of the population with respect to individual bird behaviour.

The lack of a significant difference in female visit rates before and after trapping supported the assumption that behaviour during the DLW measurement period was largely normal at the time of observation. However, an effect may not have been detected because of low statistical power due to small sample size and/or the variability of the behaviour. The practical difficulties (and costs) of the use of DLW in free-living animals has undoubtedly contributed to problems with small sample sizes, and hence low statistical power, in assessments of behavioural effects (e.g. Zurowski & Brigham 1994, Wilson & Culik 1995). The recent study by Schultner *et al.* (2010) was designed to address these problems and showed an effect of the two-sample procedure on both behaviour and estimates of energy expenditure in Black-legged Kittiwakes. The authors suggested that the initial period of restraint required for equilibration by this method may have been a major factor, acting through stress and/or by increasing time required for plumage maintenance. However, in Northern Fulmars, the behaviour of birds released immediately after injection (and without blood sampling at this stage) was as disturbed as those sampled after a two hour equilibration period (Furness & Bryant 1996).

Whatever the mechanism, an effect of stress seems likely in that Schultner *et al.* (2010) found stronger negative effects in a year of unfavourable foraging conditions; thus the kittiwakes may have had less tolerance to the stress imposed by the procedure during a year when environmental stress was already high. Experience from our work with both Great Tits and Blue Tits using a two-sample DLW method concurs with this; desertion occurred in parkland and small woods (poor quality foraging habitat, Hinsley *et al.* 2008, Hinsley *et al.* unpubl. data), but not in large woods (of a total of 95 initial trappings, 4 birds deserted and two further broods died probably because the female did not roost in the box overnight after trapping) and only occurred in the two

(out of three) years in which weather conditions were poor during chick rearing. One parkland Blue Tit which deserted in poor weather in 2005 (brood size = 4; mean chick mass = 5.3 g) had been successfully sampled, and fledged her brood, in good weather in 2004 (brood size = 7; mean chick mass = 7.7 g).

Intrinsic stress may also play a role in that birds which appeared to be naturally nervous (Drent *et al.* 2003, Careau *et al.* 2008) were amongst those least likely to be retrapped, If such birds are also likely to take a relatively long time to resume feeding young after manipulation, as found in six out of 12 Marsh Tits by Nilsson (2002), failure to retrap these birds would reduce measurement error, but would also fail to sample this subset of the population. Response to stress may also vary with species life history characteristics. For long-lived (K selected) species, abandonment of a particular nesting attempt following disturbance (perceived as threatening future fecundity) may be a more viable option than for a short-lived (r selected) species. Four of the Northern Fulmars in the study of Furness and Bryant (1996) were not seen to return to their nests within 150 hours and may have deserted. Disturbed behaviour was also reported in Northern Gannets *Sula bassana* (Birt-Friesen *et al.* 1989) and a high incidence (up to 100%) of nest abandonment in Wilson's Storm Petrels *Oceanites oceanicus* (Obst *et al.* 1987).

We did not attempt to measure the time taken by females to resume feeding because of the risk of an observer causing further disturbance; Nilsson (2002) recorded nest visits remotely using transponders glued to the birds' rings. For the three manipulated female Great Tits not seen to feed the young during the Day 2 observations, the lengths of time elapsed from trapping to observation were 2.4, 4.7 and 7.4 hours, suggesting that a delay in resuming feeding by some birds may affect their energy expenditure as found for Marsh Tits. However, it is also possible that the birds' apparent absence was influenced by the presence of the observer. For Marsh Tits, in addition to a delay in resuming feeding, the visit rates of the delayed birds were significantly

lower (on average by 9.5 visits h^{-1}) after resumption than prior to manipulation, whereas visit rates of female Great Tits in our study did not differ significantly before and after manipulation. The absence of a correlation between female visit rate and the time between trapping and Day 2 observations also indicated that feeding behaviour, once restarted, was normal after manipulation. Thus the response to DLW manipulation is likely to differ between species as well as between individuals, and to interact with environmental conditions.

Reasons for the increase in male visit rates on Day 2 are unclear, but the similar responses of both experimental and control males suggested that the birds were reacting to the presence of the observer. The increase might have been some sort of nest defence response – increased attentiveness in preparation for the possibility of defending the nest (and/or female) from a potential predator. In a study of perception of risk, Gosler (2001) showed that Great Tits responded to being trapped in a manner consistent with an increased perception of predation risk. The positive correlation between experimental male, but not female, Great Tit post-trapping visit rates and the length of time between trapping and the Day 2 observations might also indicate a nest defence response, i.e. the male becoming more alert at the time of day when the female was first trapped. However, it might also be an artefact of high variability in a small data set.

In a study of Green-rumped Parrotlets, Siegel *et al*. (1999) found post-injection visit rates to be higher (mean $\pm$ SE = 0.65 $\pm$ 0.11 visits/h) than pre-injection rates (0.50 $\pm$ 0.12 visits/h). At $P =$ 0.052 (Wilcoxon signed-ranks test, n= 18) the difference was not strictly statistically significant, but in ecological terms could merit further attention. The authors suggested that the result might be "an unlikely consequence of researcher disturbance" because the rate increased rather than decreased. Although our nested ANOVAs found no significant evidence for an effect of treatment on the increase on Day 2 for our Great Tits, the results (day x treatment interaction, $P =$ 0.13, Table 4b) also suggested that further work (with larger sample sizes) on such disturbance

effects could be of interest. The experimental males were certainly aware of the absence of their partners and could often be heard calling for them whilst the females were being processed.

The results reported here showed that the application of the DLW technique did affect Great Tit behaviour, but probably did not compromise the measurement of female energy expenditure for most birds. However, for some individuals, delayed resumption of normal feeding activity after manipulation is possible (Moreno *et al*. 1995; Nilsson 2002), but this response may differ between species, as well as between individuals. It should also be stressed that these results are for Great Tits feeding young in woodland, the response of Great Tits at other breeding stages (e.g. during incubation when they are known to be more sensitive to disturbance) and in other habitats (e.g. public parks where they are less nervous of people, and easier to both trap and retrap, pers. obs), and that of other species, may differ. The possibility of a change in behaviour of an unmanipulated member of a breeding pair, and of a difference between the sexes in response, should also be considered.

Trapping birds at night, or near the end of the day, might reduce the impact of a recovery period on parental activity, but retrapping may be more difficult for birds which do not roost in the box the next night (Tinbergen & Dietz 1994, Tinbergen & Verhulst 2000). If changes in female behaviour are partly due to muscle soreness from sampling and/or handling then detrimental effects may persist into the following day. Working in the field at night may also involve considerable practical difficulties. A 48, rather than 24, hour interval between initial and final sampling would dilute the effects of any short term changes in behaviour and maintain the advantages of working in daylight. The use of intraperitoneal, rather than intramuscular, injection would also avoid muscle soreness. Great Tits in our study showed little or no reaction when injected, there were no apparent adverse effects (the injection site was not visible in retrapped birds) and in breeding females, the bare brood patch facilitated location of the target area. The

single-sample technique, where initial isotope enrichment is calculated using an estimate of body water pool size, rather than a direct measure (Speakman 1997), avoids the initial period of restraint required for equilibration which appeared to contribute significantly to stress in kittiwakes (Schultner et al. 2010) and has been used successfully in other studies (e.g. Weathers & Sullivan 1993, Webster & Weathers 1989). Thus the potential loss of precision must be weighed against the likelihood of adverse behavioural responses which are likely to vary between individuals and species and with environmental conditions. Reporting of retrap rates, and of any adverse behavioural reactions, in DLW studies, especially in relation to species, sex, habitat type and the presence of other potential stressors, would be useful.

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Table 1. Nest visit rates of female and male great tits feeding young before (Day 1) and after (Day 2) the experimental females were trapped at the box to measure their energy expenditure using doubly labelled water. Experimental females of pairs 4 and 7 (in bold) were present, but not retrapped. Control birds were not trapped. Visits where the birds could not be sexed were not included in the calculation or analysis of visit rates. Time elapsed from trapping until the beginning of observations on Day 2 was measured from the return of the female to the box.

	Females: no. visits, h⁻¹		Males: no. visits, h⁻¹		No. visits where sex unknown		Time from trapping to
Pair	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 2 obs., h
EXPERIMENTAL BIRDS							
1	2.0	0.0	12.4	12.0	0	0	2.4
2	2.8	0.0	2.4	14.8	1	0	7.4
3	3.2	5.6	9.6	16.0	0	0	6.7
4	**3.6**	**0.0**	**10.8**	**17.4**	**7**	**0**	**4.7**
5	5.6	5.5	10.8	26.0	3	0	5.4
6	6.0	5.6	9.2	15.2	0	0	5.8
7	**7.2**	**1.6**	**8.4**	**10.8**	**4**	**0**	**5.5**
8	12.0	6.6	21.6	37.8	0	0	22.0
9	13.2	16.8	13.6	25.8	9	0	6.3
10	13.6	9.6	13.2	15.0	0	0	1.9
11	13.8	26.6	16.8	30.9	2	0	18.5

CONTROL BIRDS

1	4.2	4.8	12.0	24.0	0	0	-
2	4.8	15.6	4.8	9.6	41	9	-
3	5.4	7.8	6.0	9.6	2	3	-
4	7.3	3.3	10.0	11.3	0	1	-
5	20.2	13.0	8.2	11.0	0	0	-
6	20.4	19.2	3.6	3.6	0	0	-

Table 2. Mean visit rates on Day 1 and Day 2 for experimental (n =11) and control (n = 6) male and female Great Tits feeding young (standard errors (se) are those for comparing treatments, and not for sexes or days).

Sex	Day	Mean (se) visit rate, h^{-1}		Difference	P
		Experimental birds	Control birds	(exptl. – con.)	
Male	1	11.7 (1.5)	7.4 (1.3)	4.3 (2.0)	0.048
	2	20.2 (2.6)	11.5 (2.8)	8.7 (3.7)	0.042
Female	1	7.6 (1.4)	10.4 (3.2)	-2.8 (3.5)	0.44
	2	7.1 (2.5)	10.6 (2.6)	-3.5 (3.6)	0.34

Table 3. Results of nested ANOVA for the effects of treatment (manipulated for DLW or control), sex and observation day (Day 1 or 2) on visit rates of Great Tits. *F*-test for effects of sex and sex × treatment interaction based on sex × pair (within treatments); *F*-test for effects of day and day × treatment based on day × pairs (within pairs); *F*-test for effects of day × sex and day × sex × treatment based on day × sex × pairs (within treatments).

Source of variation	df	SS	MS	*F*	*P*
Between pairs:					
Treatment	1	41.08	41.08	0.44	0.52
Within treatments	15	1407.29	93.82		
Between birds within pairs:					
Sex	1	461.76	471.76	8.68	0.010
Sex × Treatment	1	359.72	359.72	6.76	0.020
Sex × Pair	15	798.14	53.21		
(within treatments)					
Between days within birds:					
Day	1	189.78	189.78	9.50	0.008
Day × Sex	1	215.31	215.31	21.45	<0.001
Day × Treatment	1	12.79	12.79	0.64	0.44
Day × Sex × Treatment	1	24.50	24.50	2.44	0.14
Day × Pairs	15	299.60	19.97		
(within treatments)					

Day x Sex x Pairs	15	150.57	10.04
(within treatments			
Total	67	3960.55	

Table 4. Results of nested ANOVAs for the effects of treatment (manipulated for DLW or control) and observation day (Day 1 or 2) for females and males separately.

Source of variation	df	SS	MS	*F*	*P*
(a) Females					
Between birds:					
Treatment	1	78.83	78.83	1.02	0.33
Birds within treatments	15	1154.23	76.95		
Between days within birds:					
Day	1	0.40	0.40	0.025	0.88
Day × Treatment	1	0.94	0.94	0.060	0.81
Day × Pairs	15	236.69	15.78		
(within treatments)					
Total	33	1471.10			
(b) Males					
Between birds:					
Treatment	1	321.97	321.97	4.59	0.049
Birds within treatments	15	1051.21	70.08		
Between days within birds:					
Day	1	404.69	404.69	28.44	<0.001
Day × Treatment	1	35.35	35.35	2.55	0.13
Day × Pairs	15	213.47	14.23		
(within treatments)					
Total	33	2027.69			