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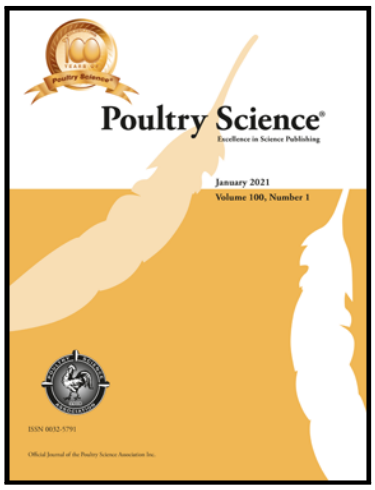
Published in:
Poultry Science

DOI:
10.1016/j.psj.2022.101838

Print publication: 01/05/2022

Document Version
Version created as part of publication process; publisher's layout; not normally made publicly available



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PII: S0032-5791(22)00145-6
DOI: https://doi.org/10.1016/j.psj.2022.101838
Reference: PSJ 101838

To appear in: *Poultry Science*

Received date: 13 December 2021
Accepted date: 2 March 2022

Please cite this article as: Laura M. Dixon , Ian C. Dunn , Sarah Brocklehurst , Laurence Baker , Tim Boswell , Sarah D. Caughey , Angus Reid , Victoria Sandilands , Peter W. Wilson , Richard B. D'Eath , The effects of feed restriction, time of day and time since feeding on behavioral and physiological indicators of hunger in broiler breeder hens, *Poultry Science* (2022), doi: https://doi.org/10.1016/j.psj.2022.101838

The effects of feed restriction, time of day and time since feeding on behavioral and physiological indicators of hunger in broiler breeder hens

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Appropriate Scientific Section: Animal Well-Being and Behavior

Abstract

Broiler breeder chickens are commercially feed restricted to slow their growth and improve their health and production, however, there is research demonstrating that this leads to chronic hunger resulting in poor welfare. A challenge in these studies is to account for possible daily rhythms or the effects of time since last meal on measures relating hunger. To address this, we used 3 feed treatments: AL (ad libitum fed), Ram (restricted, fed in the morning) and Rpm (restricted, fed in the afternoon) to control for diurnal effects. We then conducted foraging motivation tests and collected home pen behavior and physiological samples at 4 times relative to feeding throughout a 24 h period. The feed treatment had the largest influence on the data, with AL birds weighing more, having lower concentrations of plasma NEFA, and mRNA expression of AGRP and NPY alongside higher expression of POMC in the basal hypothalamus than Ram or Rpm birds ($P<0.001$). R birds were more successful at and had a shorter latency to complete the motivation test, and did more walking and less feeding than AL birds in the home pen ($P<0.01$). There was little effect of time since last meal on many measures ($P>0.05$) but AGRP expression was highest in the basal hypothalamus shortly after a meal ($P<0.05$), blood plasma NEFA was higher in R birds just before feeding ($P<0.001$) and glucose was higher in Ram birds just after feeding ($P<0.001$), and the latency to complete the motivation test was shortest before the next meal ($P<0.05$). Time of day effects were mainly found in the difference in activity levels in the home pen when during lights on and lights off periods. In conclusion, many behavioral and physiological hunger measures were not significantly influenced by time of day or time since the last meal. For the measures that do change, future studies should be designed so that sampling is balanced in such a way as to minimise bias due to these effects.

Keywords

broiler breeder, hunger, behavior, physiology, welfare

INTRODUCTION

Many animals used in commercial food production are regularly feed restricted to decrease growth rates and maintain good physical and reproductive health (review by D'Eath et al., 2009). This restriction is especially severe in the growing phase of broiler breeders, the parent stock of broilers (meat chickens). Broiler breeders share the same fast growth potential as their offspring and if fed ad libitum, these birds would have high mortality, lameness, metabolic issues and poor reproduction (Renema and Robinson, 2004). To combat this, broiler breeders are feed restricted up to about 32-33% of what they would choose to eat given free access (De Jong et al 2002) and although broiler breeder genetics will have changed since this publication, increased growth selection for broilers (e.g. Havenstein et al., 2003) will lead to even more severe restriction needed in the parent stock. This chronic feed restriction leads to the welfare concern that they are chronically hungry (reviewed by Mench, 2002; D'Eath et al., 2009). Feed restricted broiler breeders show increased activity and foraging behavior and perform abnormal or stereotypic behaviors such as pacing, spot pecking and polydipsia as well as a high motivation to access feed when available (Savory and Maros, 1993; Hocking et al., 2001; Sandilands et al., 2005; Dixon et al., 2014). Finding methods to increase satiety while maintaining slow growth could improve the welfare of millions of broiler breeders in the UK alone (Sandilands et al., 2006).

In previous research we found that feed restricted broiler breeder hens were more motivated to access an area to forage for food (appetitive feeding behavior) (Dixon et al., 2014) and they had higher levels of agouti-related protein (AGRP) mRNA in the basal hypothalamus (thought to be representative of current hunger and metabolic state) (Dunn et al., 2013b) than birds of the same age fed larger portions or ad libitum, adding to the evidence that these birds are chronically hungry. However, a criticism of this work is that the data were collected after restricted birds had run out of food, those on larger portions may or may not have had food

left, and that ad libitum fed birds had access to food until they underwent behavioral testing or were killed for physiological sampling. This may have resulted in behavioral and physiological differences in our measures depending on the time of the day data were collected, and the associated time since the last meal.

Daily oscillations in physiological and behavioral measures are known to occur (e.g. Machado et al., 2015). For example, hens are motivated to access nestboxes prior to oviposition and will display nest seeking and inspection behaviors that are not present at other times of the day (Duncan, 1989; Appleby et al., 2004). Circulating glucocorticoids are higher during the active period of animals, including broiler breeder chickens (de Jong et al., 2001) and tend to show a peak at the beginning of the activity period (Chung et al., 2011). From a feeding behavior point of view, most animals establish daily feeding rhythms when given ad libitum access to food. Free-fed domestic fowl tend to eat more at the beginning or end of the light period but less in the middle of the day (Savory, 1980). However, food-restricted animals consume food immediately after being provided access to it, while in ad libitum animals, feeding is related to time since last meal. For example, broiler breeders on a commercial level of feed restriction (from 25-51% of what they would choose to eat ad libitum) and those fed twice this amount were more motivated to work for feed by pecking a disc for a food reward than birds fed ad libitum on the same diet. Additionally, when restricted birds were compared to ad libitum birds who had feed withdrawn for 3-72h, the restricted birds did not significantly vary their number of responses throughout the day while ad libitum birds increased their responses as time since last meal increased (Savory et al., 1993). Therefore, time of day and/or time since last meal may have affected the responses of ad libitum birds in our previous motivation tests (Dixon et al., 2014) but effects on the restricted-fed birds may be minimal.

Prior research on daily rhythms of AGRP gene expression is conflicting: there was no effect of time of day on hypothalamic AGRP mRNA levels in Siberian hamsters (Ellis et al., 2008) but there was a diurnal rhythm of AGRP mRNA found in rats, with a peak 4 hours after lights off and a trough at 4 hours after lights on which was thought to be consistent with a day-night food intake rhythm of this nocturnal animal (Lu et al., 2002). Free feeding mice also had an increase in AGRP neuron electrical activity related to nocturnal feeding behavior, with less activity around dawn than later in the photoperiod when it was some time since they last fed. While in food-restricted mice AGRP neuron activity dropped as food became available but still stayed at higher levels than in freely-fed mice (Mandelblat-Cerf et al., 2015). In birds, Japanese quail fasted for 24 hours had higher AGRP mRNA compared to ad libitum-fed individuals (Philips-Singh et al., 2003), and AGRP mRNA decreased in broiler breeder hens released from a period of feed restriction and ad libitum fed for 2.5 days, suggesting expression can change relatively quickly (Dunn et al., 2013b; Caughey et al., 2018). This indicates that the time of day or the time since the last meal, especially with food restriction, could affect AGRP mRNA levels and may influence results depending on when the samples were collected.

Other gene products in the arcuate nucleus of the hypothalamus are also thought to be important in regulating energy balance through feeding stimulation or inhibition. Neuropeptide Y (NPY) is co localised and acts similarly to AGRP by stimulating feeding behavior and by its gene expression being increased in response to food restriction. Broiler breeder males reared on a commercial restriction program had significantly higher NPY gene expression than similarly aged birds fed ad libitum (Boswell et al., 1999) and feed intake can be stimulated in broilers when NPY is injected into the brain (Kuenzel et al., 1987). Pro-opiomelanocortin (POMC) neurons are anorexigenic, having a catabolic effect on energy balance, and would, when activated, be expected to decrease feeding behavior in an opposite,

inhibitory manner compared to AGRP. However, food deprivation studies in birds do not always follow this pattern. During short term food deprivation (24-48 h) and chronic food restriction (7 days) broiler chicks and layer chicks had decreased POMC expression compared to when they were fully fed (Hen et al., 2006; Higgins et al., 2010; Lei and Lixian, 2012; Fang et al., 2014) but there was no change in POMC mRNA levels in Japanese quail and broiler chicks after short term food deprivation and no change in broiler breeder hens after chronic food restriction (6 weeks) (Philips-Singh et al., 2003; Song et al., 2012). There is not much currently known about the diurnal rhythms of POMC in birds but in proestrous female rats, levels of POMC mRNA increased in the morning with a peak between 0300-1000 and then decreased by 2300 (Wise et al., 1990) and male ad libitum fed rats had a peak around midnight which decreased from 0600-1900 (Chen et al., 2004). In mammals, cocaine and amphetamine regulated transcript (CART) is also anorexigenic and involved in regulating food intake and body mass. Less is known about CART and its co-expression with POMC in birds. However decreased expression of CART mRNA and reduced immunoreactive CART fibres have been observed after fasting or food restriction in broiler and layer chickens and in zebra finches, consistent with an anorectic action of these neurons in birds (Cai et al., 2015; Singh et al., 2016; Caughey et al., 2018).

Aside from the above mentioned neurons, there are peripheral peptides which may also impact on hunger/satiety. In a complementary paper where we quantified gene expression of peptide YY (PYY) and pancreatic polypeptide Y (PPY) utilising the same samples featured in this study, we observed significant effects of time since feeding only for PYY mRNA in the pancreas. However, there were clear treatment effects with gene expression of PYY and PPY both being higher in the pancreas of ad libitum-fed birds (Reid et al., 2017). NPY neurons are also present in the gut and inhibit electrolyte and water secretions and the motility of the gastrointestinal tract (Cox, 2007). There is currently no evidence that NPY in

the gut is influenced by hunger or time since feeding but as PYY and PPY did change in the Reid et al (Reid et al., 2017) paper, it is possible that NPY, which is part of the same family, may as well. In chickens, circulating insulin levels are correlated with food intake levels (Simon, 1989) and direct injection of insulin can increase food intake (Honda et al., 2007); however insulin levels did not differ between selected lines of lean and fat birds when both were food restricted (Simon, 1989). Insulin injections also increased gene expression of POMC in chickens but did not inhibit AGRP mRNA and did not consistently inhibit NPY mRNA as it did in similar lab rat studies (Porte, Jr et al., 2002; Honda et al., 2007; Shiraishi et al., 2008). Exogenous cholecystokinin (CCK) inhibits food intake (Dunn et al., 2013a) but CCK receptor type A (CCKAR) is less abundant in chickens bred for fast growth, like modern broilers and broiler breeders, leading to a decreased sensitivity to its satiating effects (Honda, 2016). Several different mRNA transcripts are transcribed from the chicken glucagon gene that undergo tissue-specific processing to produce glucagon (GCG) in the pancreas and glucagon-like peptides-1 and -2 (GLP-1 and GLP-2) in the intestine and brain (Honda, 2016). Both GCG itself and GLP-1 inhibit food intake when injected into the brain (van der Wal et al., 1999). Levels of non-esterified fatty acids (NEFA) and glucose in the blood plasma can indicate metabolic rate and the storage or use of energy substrates (Scheurink et al., 1996). NEFA levels were increased in broilers subjected to short term food restriction (de Jong et al., 2003) but were decreased in broiler breeders subject to high levels of chronic food restriction (similar to commercial restriction levels) compared to birds who were still chronically restricted but at a less severe level and ad libitum fed breeders, while glucose levels were not affected by the different restriction levels (from ad libitum up to a restriction of 25% of the ad libitum food intake) (Renema and Robinson, 2004).

Clearly there are still gaps in our understanding of how these peptides interact to regulate feeding in chickens with even fewer studies exploring the diurnal rhythms of these peptides.

In future studies, we plan to feed broiler breeders restricted diets of different compositions that may decrease hunger and improve satiety which may lead to the birds showing more similarities to ad libitum fed birds. Therefore, we need to determine the daily rhythms and influences of feeding times for our key measures to ensure future results are not influenced by these outside factors. This study was specifically set out to ensure feeding-driven changes were discernible from any photoperiod- or circadian-driven cycles. Additionally, these results from a well powered study may help to improve our understanding of the regulation of energy balance in chickens and what potential changes occur in relation to time of day and hunger status. Therefore, this study aimed to determine how behavior, appetitive feeding motivation, AGRP mRNA in the basal hypothalamus and other neurobiological and physiological measures vary with time after feeding, whilst controlling for effects relating to time of day for restricted and ad libitum-fed broiler breeders. We hypothesized that restricted-fed birds would show the lowest behavioral and physiological measures relating to hunger shortly after a meal and the highest shortly before a meal, with other time points giving intermediate results, and that restricted-fed birds would always show behavioral and physiological signs of increased hunger compared to ad libitum-fed birds.

MATERIAL AND METHODS

Ethical Considerations

Food restriction is likely to result in hunger, but welfare issues which are typical in commercial farming need to be replicated in the laboratory so they can be studied for potential solutions. The levels of food restriction we imposed were similar to those used routinely in the poultry industry, while 1 feed treatment was ad libitum access to feed. Ad libitum feeding of broiler breeders from hatch can cause welfare concerns (Renema and Robinson, 2004); therefore our birds did not begin the ad libitum feeding treatment until they

reached 7 weeks of age and the experiment was ended when birds were 12 weeks old, at which age they were still active and healthy. All procedures in this experiment were carried out under Home Office Licence and with the SRUC Animal Experiment Committee's approval; birds were inspected a minimum of 3 times per day.

Animals and Housing

216 non-beak-trimmed Ross 308 broiler breeder female chickens (Aviagen, Stratford, UK) were raised from 1 day-old chicks in 2 separate batches, 6 weeks apart (108 chicks per batch). Each batch was housed in 2 rooms, with 12 floor pens with wood shavings (1.0 × 1.5 m) in groups of 9 birds per pen. The lighting schedule for the first day was 23.5L:0.5D hours light:dark after which the photoperiod was gradually reduced to 8L:16D over 10 days. Temperature followed commercial recommendations, decreasing from around 30°C at bird level at 1 day old to around 20°C by 4 weeks of age. Chicks were given ad libitum water from bell drinkers and were fed chick starter crumbs for the first 3 weeks, chick starter pellets for the following 3 weeks and then grower pellets from the beginning of 6 weeks of age to the end of the trial (all ABN, Cupar Mills, Fife). The feed formulations were developed in consultation with a broiler breeder producer and feed manufacturer to be in line with commercial broiler breeder standards and are proprietary, however all diets met the National Research Council requirements. Food was provided ad libitum for the first 7 days and then in restricted amounts given at 9:00 h each day that were gradually increased from 26 to 44 g per bird per day by the beginning of the 6^{th} week, as per the Ross 308 parent stock guidelines (Aviagen, 2013). At 2 weeks of age, all birds were weighed and wing tagged (10 mm × 10 mm padlock-style tags, Roxan Developments Ltd., UK).

At 6 weeks of age, all birds were weighed and regrouped into pens of 9 birds according to matched body weight. The photoperiod was also increased from 8L:16D to 10L:14D hours at this point to allow sufficient hours of light to complete all the necessary training and testing. All birds were weighed about weekly from 2 weeks of age to the end of the trial (12 weeks of age).

Experimental Design

Pens were in 4 spatial blocks across both rooms in each batch with 3 pens of similar average weight making up each block. In order to optimise balance of feed treatments with average pen weight, the 3 different feed treatments (Ram, Rpm and AL) were allocated at the pen level within each block using 2 3x3 latin squares, 1 per batch, plus the addition of a random allocation to the remaining 3 pens in 1 block in batch 1, which was reversed for the remaining block in batch 2. This resulted in 8 pens and 72 birds in each feed treatment over both batches (Fig 1). Birds within pens were allocated to be culled for post mortem at 4 times relative to feeding (see below), randomly allocating the 4 lightest and the 4 heaviest in each pen to the 4 times, and then randomly allocating the remaining 4 birds per treatment in each batch to the 4 times. Birds within pens were allocated to 1 of 3 scheduling groups for which motivation tests were staggered by 1 week, in such a way that each scheduling group contained equal numbers of birds per batch in each feed treatment by post mortem time relative to feeding. Allocation of the 12 birds of each diet in each scheduling group to 1 of 3 sets of apparatus (see below) was achieved by using 2 3x3 latin squares, 1 for each batch. This ensured that scheduling group by apparatus was balanced with feed treatment by post mortem time relative to feeding. Similar approaches were used to ensure balance between each feed treatment by post mortem time relative to feeding whilst also optimising balance with bird

242 weight for the 3 post mortem teams and 2 days on which post mortems were carried out per
243 batch, the 3 laboratory processing days per batch, the 2 testers carrying out the foraging tests
244 and order of sampling for all the various measurements.

245 ***Treatments and Times of Measurements***

246 2 treatment groups of 72 birds (8 pens) each were fed the standard commercial restricted diet
247 (R) which was provided to the birds either first thing after lights came on in the morning at
248 07:00 h (Ram) or at 16:00 h (Rpm) which was 1 hour +/- 15 mins before lights went off in
249 the evening (17:00 h). A third treatment group of birds were fed the commercial diet ad
250 libitum (AL). Behavioral and physiological measures (see below) were collected throughout
251 various 24-hour periods, once after the birds had eaten (minimum time since being fed), once
252 before the next feeding (maximum time since being fed) and at various other time points
253 between the minimum and maximum (see Fig 2). Birds had been allocated to be culled for
254 post mortem during ~2 hour intervals starting at 1, 7, 16 and 22 hours relative to the feeding
255 time. These specific times were chosen in order that the circadian time of sampling was as
256 similar as possible between Ram and Rpm birds and in order that there were equal sampling
257 points during lights on and lights off. AL birds were fed and sampled at the same time as
258 Ram birds. Home pen scan sessions were chosen to also coincide with the time in the day
259 birds were culled for post mortem, plus the addition of 1 session in the middle of the day, but
260 all birds were observed at all 6 sessions during the day regardless of the time when they were
261 to be culled for post mortem. Foraging tests took place over intervals of 2 hours whilst home
262 pen scan sessions were 1 hour long (see Fig 2).

263 ***Behavior Tests***

264 ***Foraging Motivation Test.*** *Apparatus – set up, habituation and training.*

The foraging motivation apparatus and habituation and training procedures have been described previously (Dixon et al., 2014), but in brief the apparatus consisted of a wooden start platform which had a ramp into a runway which could be filled with varying depths of water and led to a moveable wooden platform where wood shavings were placed during testing (wood shavings platform). The apparatus was covered by a lid that prevented the birds from flying across the runway to avoid water during training and testing.

Before training began, birds were habituated in groups to the apparatus with no water or wood shavings for 3 15-minute sessions. Birds then received 2 individual habituation sessions in the apparatus as training and testing were done on an individual basis.

Training began at 6 weeks of age, coinciding with when the diet treatments began, and took 1 week. There were 3 training stages. First the birds were placed in the apparatus with the 2 wooden platforms directly next to each other (no ramps), wood shavings were present on the wood shavings platform and birds were given 10 minutes to move from the start to the wood shavings platform. Next the wood shavings platform was moved 1 m from the start platform and the ramps were added back in. No water was in the runway and again birds were given 10 minutes to reach the wood shavings platform. Finally, this step was repeated but with enough water in the runway to just cover the birds' feet (about 20 mm). Birds did not progress to the next training stage until they had successfully completed the previous one.

Testing. Each batch of birds was divided into 3 groups with each group being tested for 1 week. Birds were each tested 4 times, once per day for 4 consecutive days, with the 12 birds from each of the 3 diet treatments tested on 1 of the 3 apparatuses (see above). The test time interval for each bird was selected to match the time relative to feeding when they were to be culled for post mortem apart from those culled around midnight for which foraging tests were instead at 17:00-19:00 h (Ram) or 05:00-07:00 h (Rpm). From previous experience,

289 birds disturbed mid-way through the dark period would not perform well in a test
290 environment and would merely rest, thus not giving accurate data for this test. This
291 arrangement resulted in all birds being tested either 1-3, 7-18 and 22-24 hours since last feed
292 and all tests conducted during, or within 2 hours either side of, the period when lights were
293 on (see Fig 2). Testing began with the first group of birds when they were 8-9 weeks of age,
294 the second group when they were 9-10 weeks of age and the third group when they were 11
295 weeks of age. For birds in groups 2 and 3, a re-fresher training session (similar to the third
296 training session) was conducted to ensure they were still familiar with the apparatus. For the
297 first test, the wood shavings platform was moved 1.5 m from the start platform, with 0.8 m
298 between the bottom of the ramps and water was added to the runway. Because birds on the
299 different feed treatments grew at different rates over the test, the water depth was
300 proportional to mean leg length of the 12 birds to be tested on each apparatus in each test
301 week. To do this, the length of the birds' legs was measured from the ground to the top of the
302 hock before their test week.

303 Over subsequent tests, the 'cost' of accessing the wood shavings platform, in terms of water
304 depth and length was increased in a stepwise manner: water depth was increased in
305 increments relative to the average length of the birds' legs for each feed treatment (water
306 depth: test 1=2/6 leg length, test 2=4/6 leg length, test 3=6/6 leg length, test 4=8/6 leg
307 length). This resulted in water depth levels that ranged from 18mm at the first test to 73-
308 94mm at the 4th test. As the water depth increased with each test, the length of the runway
309 between the bottom of the 2 ramps was also increased from 0.8 m at the first test by 0.8 m
310 each time up to a length of 3.2 m at the 4th test.

311 Each test lasted about 20 minutes. At the beginning of a test, a bird was placed on the start
312 platform and could spend the test time in whatever areas of the apparatus she chose to. After
313 the 20 minutes were up, the bird was removed from the apparatus. Due to the number of birds

being tested, 3 identical apparatuses were used and 2 people took shifts placing the birds on the start platform at the beginning of each test.

Measurements. Measurements were made from videos of the foraging tests by 1 observer using The Observer XT (Version 11, Noldus, Wageningen, The Netherlands). For all tests, time spent in the different parts of the apparatus was recorded and from this whether the bird reached the wood shavings platform (defined by the bird having both feet on it) and latency to reach the wood shavings platform were derived. Behavior on the wood shavings platform was also recorded using the Observer XT giving total durations that the birds spent in the foraging area foraging, sitting, standing, walking or preening using the same behavior definitions as in the Home Pen observations (below). For tests 1 and 4 of each week, start platform behavior was also recorded to determine how the birds were using the start platform and to increase the amount of data available on the AL birds who spent most of their time on the start platform.

All birds were tested with all platform distances and water depths, even if they gave up crossing the water to reach the wood shavings in earlier tests. This allowed statistical analyses of a full complement of longitudinal data resulting in more power than would be the case for analyses of summary measures such as the maximum cost paid (distance/depth overcome) to get to the wood shavings platform.

Home Pen Observations. All pens were video recorded for 24 hr periods once a week for 3 weeks during days when foraging motivation testing was not occurring when birds were aged 9-11 weeks. Each bird in a pen was individually identified by a pattern made with black livestock marker. Scan sampling was carried out by 1 observer during 6 1-hour sessions throughout the 24 hour period, chosen to coincide with the time of day birds were to be culled for post mortem, plus the addition of 1 session in the middle of the day (see Fig 2). The

behavior of each bird in each pen was recorded for 10 scans, 6 minutes apart, for the 3 sessions during lights on and 5 scans, 15 minutes apart, for the 3 sessions during lights off. The behaviors recorded were inactive (standing/sitting/sleeping), walking (including running), foraging (pecking and scratching at litter), feeding (pecking at feed), drinking (pecking at and swallowing water), object pecking (pecking at feeder, drinker, pen walls), preening (while sitting or standing), dustbathing, aggressive pecking (peck directed to the head of another bird, delivered in a sharp, downwards manner), non-aggressive pecking (gentle and vigorous feather pecking, pecking at another bird's beak), and other (wing flap, shake, stretch, bill wipe). Walking and foraging were also combined for statistical analysis to form the category 'active behavior'.

Physiological Measures

At 12 weeks of age, blood, brain and gut tissue samples were collected from all birds. Due to the number of birds, sampling was done for each batch over 2 non-consecutive 24-hour periods and 3 teams of 3 people each were involved in the sampling during all 4 periods. The sampling times for these collections were relative to feeding times (see Fig 2). At the beginning of a sampling time, a bird was removed from their home pen, weighed and had 2 mL blood drawn from the brachial wing vein. This was split equally into 2 1.5ml microfuge tubes (Sarstedt, Leicester, UK), 1 containing 100µl 0.6M NaF/ 0.18M K Oxalate solution (for glucose measurements) and the other 50µl Heparin (1000IU/ml) (for NEFA measurements). These tubes were mixed and then stored on ice for up to 1 hour before being centrifuged at 8000g for 10 minutes at 4^{o}C and the plasma removed and stored at -20^{o}C until analysis. The bird was then euthanised with an overdose of IV pentobarbital. Once death had been confirmed, digestive organs and contents were weighed. Tissue samples (40-100 mg) were taken from the gut and immediately stored in liquid nitrogen until transfer to a -80^{o}C freezer: proventriculus (ProV), gizzard, pancreas, liver, and gallbladder. Basal hypothalamus was

363 dissected as described previously (Dunn et al., 2013b). Contents from the crop was weighed
364 and scored on appearance: 1: Empty - no liquid or solid food evident, 2: Wet mush - mainly
365 liquid with some soft solid food. , 3: Solid mush - soft solid food, 4: Mix of dry pellets/solid
366 mush - mainly soft solid food with few dry whole food pellets, 5: Dry pellets - whole dry
367 food pellets, very little or no soft solid food.

368 RNA extraction and reverse transcription and measurement of anorectic (POMC, CART) and
369 orexigenic peptide (AGRP, NPY) genes in the basal hypothalamus and genes related to
370 metabolism in the pancreas (cholecystokinin A receptor (CCKAR), NPY) were carried out by
371 RTPCR as reported previously (Dunn et al., 2013b; a) and PPY was measured as reported
372 (Reid et al., 2017). Glucagon (GCG), and Insulin (INS) were measured in the same way as
373 the other RTPCR assays using the following primers; **GCG:** Forward – 5'-
374 TGATAGTTCAAGGCAGCTGG; Reverse – 5'-AAAATCCTGAGCTCGTCTGC; **Insulin:**
375 Forward – 5'-TCCTTGTCTTTTCTGGCCCT; Reverse – 5'-
376 GCTCAACAATCCCTCGCTTG.

377 Glucose and NEFA were measured at the Easter Bush pathology lab (R(D)SVS, Easter Bush,
378 UK) on an Instrumentation Laboratory 650 analyser (Werfen, Warrington, UK) using
379 Instrumentation Laboratory and Randox Laboratories (Crumlin , N Ireland) analysis kits
380 respectively.

381 ***Statistical Analysis***

382 ***Foraging Motivation Test.*** Linear mixed models (LMM) were fitted to latency to
383 reach the wood shavings platform, and durations on the start platform and wood shavings
384 platform, calculated as a proportion of total test time (all angular transformed). LMM were
385 fitted to durations for different behaviors exhibited on the wood shavings platform for

386 successful birds and on the start platform for all birds at test numbers 1 and 4 only calculated
387 as a proportion of time spent there (all angular transformed).

388 Generalised linear mixed models (GLMM) were fitted to the binary variable whether a bird
389 successfully reached the wood shavings platform or not, with logit link function, binomially
390 distributed errors and offset by total test time (log transformed).

391 Random effects were included for batch, for individual pens of birds and individual birds, and
392 for LMM only blocks within batches and test numbers within pens, but they were all fairly
393 small apart from the variability between birds and between test numbers within birds (i.e. the
394 residual for LMMs).

395 Fixed effects were included for the 3 apparatuses, the 2 testers (main effects only) and the 4
396 test numbers, bird age (fitted as a 3 level factor), dietfeedtime (AL, Ram, Rpm) and the time
397 interval relative to feeding category (1.2-2.6, 7.2-17.5, 22.2-23.6 hours) at which birds were
398 tested and all interactions. These models were fitted to 4 different subsets of the data
399 (depending on the measurement, on availability of data, and on what was of interest): the
400 whole data set, R birds only, R birds that successfully reached the wood shavings platform
401 only or test numbers 1 and 4 only. In some cases, due to sparse and/or missing data, it was
402 necessary to obtain results from simpler fixed effects models with fewer interaction terms
403 than 4 way. For the GLMM for whether a bird successfully reached the wood shavings
404 platform, for all data only main effects were included whereas for R birds only interactions
405 up to 3 way were included. For LMMs applied to behaviours on the wood shavings platform
406 for successful birds, only interactions up to 3 way were included.

407 ***Home Pen Behavior.*** Classifications from the original ethogram of behaviors
408 statistically analysed were feeding (pecking at feed), foraging (pecking and scratching at
409 litter), drinking (pecking at and swallowing water), object pecking (pecking at feeder,

drinker, pen walls), preening, walking (including running), inactive (standing/sitting/sleeping), as well as active (walking, running or foraging classes combined). Behaviors dustbathing, aggressive pecking, non-aggressive pecking, and other occurred too rarely to be statistically analysed. For each of these classifications, the data was summarised up (over the 10 scans for lights on sessions and the 5 scans for light off sessions) into tables of counts by the classes for each bird in each session, prior to subsequent statistical analyses. So that is 18 tables per bird (3 weeks by 6 sessions per 24 hour period). These tables of counts were constructed both including the not visible class and excluding it. Initial data exploration for the 8 resulting classifications suggested that exclusion of not visible birds had no impact on the results and so results presented here exclude these scans. Initial data exploration showed that whether lights were on or off dominated behaviors, with many behavior counts very low at night, so it was necessary to analyse data separately for lights on and lights off.

In order to analyse the proportions of scans in each different behavior class GLMMs were fitted to the binomial count for that behavior class for each bird in each session with binomial total the number of scans for which the bird was visible in that session, logit link function and binomially distributed errors.

Random effects were included for batch, for individual pens of birds and individual birds, and for different weeks within pens and within birds, and for different sessions within pens and weeks (flocking behavior), and dispersion was fixed at 1. All the variance components were fairly small apart from the variability between birds and for flocking behavior for some behavior classes.

Fixed effects were included for the week of observation (a proxy for bird age), the time during lights on (8:00-10:30, 10:30-13:30, 13:30-16:00 h) or lights off (16:30-20:00, 22:30-

01:45, 4:30-7:45 h) and dietfeedtime (AL, Ram, Rpm), all fitted as 3 level factors, and all interactions. Where the data was sparse it was necessary to obtain results from simpler fixed effects models with fewer interaction terms than 3 way. Only main effects were included for feeding, drinking, foraging and object pecking when lights were off and only interactions up to 2 way were included for active (locomotion or foraging) and locomotion when lights were off and feeding when lights were on.

Physiological Measures. LMMs were fitted to bird and organ weights (log transformed), crop content weight (log plus 1 transformed), an ordinal variable for the crop content score (1: Empty, 2: Wet mush, 3: Solid mush, 4: Dry pellets/solid mush, 5: Dry pellets), blood plasma NEFA and glucose concentrations (both log transformed) and expression measures (log transformed). Expression measures were standardised by dividing by values for the housekeeping gene before calculating logs.

Random effects were included for batch, the 4 different days on which PMs were done (identical to the lab day for expression measures), each pen of birds and for LMMs only blocks of these pens within each batch, the 4 different days on which PMs were done within pens and individual birds (the residual). Fixed effects were included for the 3 PM teams (main effect only) and for bird age (fitted as a 2 level factor), dietfeedtime (AL, Ram, Rpm) and the time interval relative to feeding category (1.2-3.2, 6.9-8.7, 15.9-18.3, 22.0-23.8 hours) at which birds were tested and all interactions.

For LMMs models were fitted to all data and also to data omitting outliers (as defined by the linear mixed model residuals) to confirm that results for all data reported here are not just attributable to the outliers.

Pearson's correlation coefficient (ρ) was calculated between continuous measures.

All Statistical Analyses. Fixed effects were tested sequentially in the order given above, so, for example, effects of dietfeedtime and time relative to feeding or time in the day are tested after adjusting for effects of apparatus, tester, post mortem team, and so on. Although the experimental design ensured balance with these factors, where only a subset of data was analysed (such as behavior on the foraging platform) confounding is likely to occur so test order is important. Alternative parameterisations of the above models were fitted including fixed effects of both diet (AL,R) and of dietfeedtime (AL,Ram,Rpm), because testing dietfeedtime after diet provides an explicit test of whether there is an effect of feeding time for the R birds (i.e. tests explicitly for a difference between Ram and Rpm). This also provides explicit tests of whether there is evidence that an effect of time relative to feeding, or time in the day, differs for Ram and Rpm birds or whether significant interactions between dietfeedtime and times are just due to differences in trends between AL and R birds.

P values are based on approximate F tests when available but otherwise are based on Wald tests. Model estimates (+-SE) were obtained from the model with dietfeedtime (not diet) in the fixed effects back transformed onto the original scale to aid interpretation. Post hoc tests were carried out by using Fisher's least significant difference test for which residual degrees of freedom were the same as those used in the approximate F tests.

All data was compiled in MS Excel. Genstat 18 was used for the study design, data processing and all statistical analyses.

RESULTS

Ad Libitum Versus Restricted Diets

The feed treatment had the largest effect on all measures compared to other factors. As expected, the birds fed AL were heavier than both R treatment birds when weighed before

480 culling at 12 weeks of age (P<0.001; Table 1). Consistent with their greater body weight, AL
481 birds also had heavier gall bladders (empty), gizzards, livers, pancreas and proventriculus (all
482 P≤0.001). Correlations were highest between bird weight, and weights of liver, pancreas and
483 proventriculus (all Pearson's ρ>0.88). Averaged over sampling times, AL birds had slightly
484 higher crop content scores (indicating more recent feeding; P=0.033) and lower plasma
485 NEFA concentrations (P<0.001) than Ram and Rpm birds. Additionally, AGRP and NPY
486 mRNA levels in the basal hypothalamus were lower in AL than both R treatment birds
487 (P<0.001) while POMC and PPY mRNA were higher in AL birds (P<0.001 and P=0.002,
488 respectively). PPY results were previously reported in (Reid et al., 2017). AGRP and NPY
489 mRNA levels in the basal hypothalamus were highly correlated (Pearson's ρ=0.83), whilst
490 CCKAR, insulin and PPY mRNA levels in the pancreas were also correlated (Pearson's
491 ρ>0.64). Correlation between CART and POMC mRNA levels in the basal hypothalamus
492 was more marginal (Pearson's ρ=0.45) as was correlation between NPY and GCG in the
493 pancreas (Pearson's ρ=0.45). There was no statistically significant effect of feed quantity
494 treatment on any of the other physiological measures (P>0.05).

495 In the foraging motivation test, R birds spent less time on the start platform, were more
496 successful at completing the test (reaching the wood shavings platform), had a shorter latency
497 to reach the wood shavings platform and spent longer on it than AL birds (all P<0.001; see
498 Table 2). While on the start platform, AL birds, when compared to R, stand/sit or preen more,
499 and forage or walk less (all P<0.001), with Rpm birds performing more walking than Ram
500 birds (P=0.043). Both R treatment birds spent similar amounts of time foraging, walking or
501 standing on the wood shavings platform (P>0.05) but Ram birds spent slightly more time
502 preening (P=0.044).

503 During lights on in their home pens, averaging over time in the day effects, AL birds spent
504 more time feeding than R birds, and Ram birds drank more and did more object pecking and

spent less time being inactive than Rpm and AL birds (all P<0.001; see Table 3); although there were also significant interactions between feed treatment and time of day. AL birds also preened more and walked less than R birds (P<0.001), whilst Rpm birds preened more than Ram birds (P=0.029); however all birds performed similar amounts of foraging (P>0.05). In the lights off period, averaging over time in the night effects, Rpm birds foraged more, drank more, did more object pecking and were more active overall than the Ram and AL birds (P≤0.006). They also walked more than the Ram birds with AL birds walking the least (P=0.001). Ram birds spent less time feeding than AL and Rpm birds (P<0.001) and Rpm birds spent less time being inactive during lights off (P<0.001). However, there were significant interactions between feed treatment and time of night.

Time Relative to Last Meal

Bird weight at culling was lighter at 1-3 h and slightly heavier from 7-18 h after feeding then decreased again before the next feeding time (P=0.047; Table 4). Averaged over feed treatments, crop content was heaviest right after being fed (1-3 h) and decreased over time, being lightest right before their next feed (22-24 h ; P<0.001) and crop content scores decreased as time after feeding increased (P<0.001); although there were some significant interactions between feed treatment and time since feeding for these measures. Averaged over feed treatments, plasma NEFA concentrations decreased at 7-9 h since the last feed then increased to their highest before being fed the next meal (P<0.001) while plasma glucose concentrations were highest 1-3 h since the last feed then decreased with time maintaining the same level from 16 h since the last feed (P<0.001); although again there were some significant interactions of feed treatment and time since feeding for these measures. Of all the brain and pancreas gene expression measures, only AGRP mRNA expression in the basal hypothalamus changed with time since feeding. This was highest right after feeding, then decreased and stayed fairly constant from 7 h after feeding (P=0.028). Empty gallbladder

weights were heaviest at 22-24 h since the last feed (P=0.009) while gizzard weight decreased from 16-18 h post feeding (P=0.012). Averaged over feed treatments, liver weights were lowest at 1-3 h, and then increased at 7-18 h before decreasing at the time before the next feed (P<0.001); although there were some marginally significant interactions between feed treatment and time since feeding. There was no effect of time relative to last meal on any other physiological measures (P>0.05; Table 4).

For the foraging motivation test, averaging over feed treatments, there was no effect of time since last feeding on test success (reaching the wood shavings platform) or time spent on the start platform (P>0.05; Table 5); although there were some significant interactions of feed treatment and time since last feed. However, latency to reach the wood shavings platform decreased at 22-24 h after the last feed (P=0.028) but time since the last feed did not affect the proportion of time birds spent on the wood shavings platform (P>0.05). On the wood shavings platform (Ram and Rpm birds only in analysis) the amount of standing and walking birds performed 7-18 h hours since last feeding was less than just before their next feed (P=0.010 and P=0.012, respectively); however the amount of time spent standing and walking at 1-3 h after their last feed was not significantly different from either of these times since last feeding (P>0.05). These birds also had a corresponding peak in foraging behavior at 7-18 h since their last feed which decreased at 22-24 h (P=0.020). For behavior on the start platform, birds were found to preen and walk more (P=0.012, 0.013, respectively) and forage less (P<0.001) at 22-24 h since their last feed and stand and sit more 7-18 h since their last feed (P=0.027) compared to 1-3 h since their last feed; however standing and sitting at 22-24 h was not significantly different from either of those times since last feed (P>0.05).

In the home pen during the lights on period, averaging over feed treatments, birds decreased their drinking and object pecking (P<0.001; see Table 6) and to a lesser extent foraging (P=0.042), and increased walking and being inactive (P<0.001), with time in the day;

555 although there were some significant interactions between feed treatment and time in the day.
556 Preening had a peak around the mid-light period (P<0.001). During the dark period,
557 averaging over feed treatments, the amounts of drinking had a dip in the middle of the night
558 (P=0.003) when inactivity peaked (P<0.001), object pecking was highest just after lights off
559 (P=0.014), and walking, preening and overall activity increased shortly before the lights came
560 back on (P<0.001); although there were significant interactions between feed treatment and
561 time in the day.

562 ***Feed Treatment by Time Relative to Last Meal Interactions***

563 Crop content weight was fairly consistent for AL birds across the day, with a small peak at 7-
564 9 hours post feed top up, while crop content was heaviest at the start for both Ram and Rpm
565 birds then decreased as time since last feed increased (P<0.001; Fig 3a). Birds fed AL had a
566 fairly constant crop content score over time with a slight increase after 7-9 h post feed
567 (ranging from a score of 2.5-3) but Ram and Rpm crop content scores were higher than for
568 AL birds just after feeding and decreased as time since last feeding increased (ranging from
569 scores of 4 down to 1, P<0.001) (Fig 3b), indicating a shift from fuller, drier crop contents to
570 emptier/wetter. Plasma concentrations of NEFA also stayed fairly consistent for AL birds
571 throughout the day but NEFA increased for Ram and Rpm birds by 22-24 h since being fed
572 (P<0.001; Fig 3c). Rpm and AL birds had consistent plasma glucose concentrations while
573 glucose levels in Ram birds were higher just after being fed (1-3h) and then decreased to a
574 level similar to AL and Rpm by 7-9 h since being fed (P<0.001; Fig 3d). Both R treatment
575 birds had constant liver weights throughout the day (averaging Ram=20.8g, Rpm=22.6g,
576 back-transformed values) but AL birds had an increase in liver weight after 7 h from the last
577 feed (ranging from 55.9-73.0, back transformed values) ($F_{6, 163}$=2.34, P=0.034). For crop
578 content weight, NEFA and liver weight the interaction between time in the day of feeding for
579 R birds and the time since last feeding is not significant after adjusting for the interaction

between AL versus R birds and the time since last feeding, which confirms that the highly significant interactions are due only to differences in time since last feeding between AL and R birds and are unaffected by the time in the day of feeding for R birds. In contrast for glucose, the interaction between time of feeding for Ram and Rpm birds and the time since last feed is highly significant ($P<0.001$) after adjusting for the interaction between AL versus R birds and the time since last feed. There were no statistically significant interactions between treatment and time relative to last meal for any of the other physiological measures ($P>0.05$).

For the Foraging Test, as time since last feeding increased, AL birds maintained high levels of standing/sitting on the Start Platform, whilst Ram and Rpm birds increased their standing/sitting with time relative to feeding ($P=0.038$; Fig 4a). AL birds decreased time standing on the Start Platform whilst R birds increased time standing on the start platform with time relative to feeding ($P<0.001$; Fig 4b). AL birds spent little time foraging on the start platform whilst Ram and Rpm birds spent less time foraging with increased time relative to feeding ($P=0.003$; Fig 4c). For all these behaviors the significant differences were between the AL and R feed treatments not between the differences in feed time of Ram and Rpm birds ($P>0.05$). For the successful birds (i.e. they reached the wood shavings platform), Rpm birds showed a slight decrease with time since last feed in the amount of foraging and a slight increase in walking in relation to time since last feed, while Ram birds had a peak in foraging and a decrease in walking at 7-18 h since last feeding (foraging: $P=0.005$; Fig 4d, walking: $P=0.016$; Fig 4e). There were no significant interaction effects for any of the other motivation test measures ($P>0.05$).

In the home pen, as the daylight period progressed, AL birds increased their feeding, and Ram and Rpm birds decreased their feeding/pecking at the feeder by 10:30 h ($P=0.018$; Fig 5a). AL and Ram birds maintained constant levels of foraging and walking throughout the

day while Rpm birds decreased foraging and increased walking towards the end of the light period ($P<0.001$ for both; Fig 5b, e). AL and Ram birds also drank more consistently throughout the light period, with Ram birds drinking more than AL birds and more so at the start, while Rpm birds starting off drinking more than AL birds, then decreased their drinking to lower levels than AL birds by the end of the light period ($P<0.001$; Fig 5c). AL birds decreased their preening behavior after 13:30 h, whilst R birds maintained broadly constant lower levels of preening throughout the day ($P<0.001$; Fig 5d). Whilst Ram birds are less inactive throughout the day (Fig 5f) inactivity increased with time in the day more for R birds than AL birds ($P<0.001$) but the trend was slightly different for Ram and Rpm birds ($P=0.026$). In the dark period, AL and Ram birds increased preening and walking behaviour in the period before lights on, whereas Rpm birds decreased preening and walking mid-dark period, with preening increasing again before lights on and walking being the highest just after lights off ($P=0.002$, <0.001 respectively; Figs 6a & b). In general, AL and Ram birds were most active just before lights on while Rpm birds were most active just after lights off; although their activity levels were similar to those of the AL birds before lights on ($P<0.001$; Fig 6c). Conversely, AL and Rpm birds were least inactive just before lights on whilst Rpm birds were least inactive just after lights off ($P<0.001$; Fig 6d).

Foraging Test Increase in Cost

The proportion of R birds successfully reaching the wood shavings platform decreased with tests 3 and 4 (range mean±SEM estimated from GLMM: test 1 (63%,79%), test 2 (66%,82%), test 3 (45%,65%), test 4 (30%,48%), $Wald_3=14.48$, $P=0.002$). AL birds maintained a high latency to reach the wood shavings platform throughout the 4 tests ($P<0.001$) while the latency for Rpm increased in test 4 and Ram had a decreased latency in test 2 which increased again in tests 3 and 4 ($P=0.001$; Fig 7a). AL birds consistently spent the majority of all tests on the start platform and little time on the wood shavings platform

whilst R birds only spent about 50% of test time on the start platform (Fig 7b) and around 10% of test time on the wood shavings platform (Fig 7c). More variation between test numbers was seen for R than AL birds on the start platform (P=0.023; Fig 7b) and on the wood shaving platform (P=0.016; Fig 7c), with R birds generally spending less time on the wood shavings platform with increased test number. Although the trend with test number of time spent on the start and wood shaving platforms differed for Ram and Rpm this was not statistically significant (P>0.05). The amount of preening and walking behaviour on the start platform remained consistent for tests 1 and 4 for AL birds, whilst preening behavior increased in test 4 compared to test 1 for R birds (P=0.017; Fig 7d) and walking decreased (P=0.022; Fig 7e). These effects were more apparent for Ram birds although tests indicated no significant difference in behaviour on the start platform between Ram and Rpm birds (P>0.05). There were no significant interactions between feed treatment and test number for any other foraging motivation test measures (P>0.05).

Other Factors Influencing Results

There were other factors in the design of the experiment and processing of samples that influenced the results. For example, the amount of time spent feeding in the home pens during lights on decreased in week 3 (bird age 82 d) compared to the other test weeks (1, bird age 63-68 d and 2, bird age 69-75 d; P<0.001; Supplementary Tables 1a&b). From the 3 teams collecting data during post mortem sampling, higher plasma glucose levels were recorded from samples collected by Team C than by Team B (P=0.009; Supplementary Tables 2a&b) with Team A intermediate. Higher AGRP and POMC values were measured in tissues dissected by Team A than those for the other teams (P≤0.001). Birds had a shorter latency to reach the wood shavings platform when tested in apparatus 3 compared to identically designed apparatuses 1 and 2 (back-transformed means - apparatus 1: 1093 s, apparatus 2: 1040 s, apparatus 3: 842 s, $Wald_2$=7.17, P=0.028) and for 1 of the testers (back-

655 transformed means – tester LB: 950 s, tester LD: 1049 s, $Wald_1$=4.42, P=0.036). Birds also
656 spent a smaller proportion of the test time on the start platform standing in apparatus 2
657 compared to 1 and 3 (back-transformed means – apparatus 1: 0.67, apparatus 2: 0.41,
658 apparatus 3: 0.59, $F_{2, 174}$=3.68, P=0.027) and a larger proportion of the test time walking on
659 the start platform in apparatus 2 compared to 1 (back-transformed means: apparatus 1: 0.020,
660 apparatus 2: 0.034, apparatus 3: 0.027, $F_{2, 180}$=4.45, P=0.013). While these results are
661 interesting and important in relation to experimental design and balancing, these factors were
662 not the main objectives of this experiment, so the full details of these results have been
663 included as online supplementary materials.
664

665 **DISCUSSION**

666 ***Time Relative to Last Meal***

667 The aim of this study was to determine what effects time since last feeding had on behavioral
668 and physiological measures relating to feed intake and hunger while accounting for time of
669 day in restricted and ad libitum fed broiler breeders. For the many of measures there was no
670 evidence of effects related to the time since last feed from this study, e.g. NPY, POMC and
671 CART gene expression, pancreas weight, foraging test success, proportions of time spent on
672 the start and wood shavings platforms. Additionally home pen behaviour was highly
673 influenced by light/dark status, not time relative to last meal, leading to these measures being
674 analysed separately for the lights on and lights off periods.
675 However some measures did show changes: AGRP mRNA expression was highest after
676 being fed then decreased and maintained a consistent level from 7-9 hours post feed. At first
677 sight, this is an unexpected finding, since in previous work, higher levels of AGRP are
678 associated with feed restriction over the longer term. The high levels may suggest a lag
679 between the activity of the AGRP neurones and the expression of AGRP as well as the need

for the nutrient signals to be translated into satiety signals which can be read by the orexigenic second order neurones in the brain. It may also reflect the fact that AGRP seems to be involved with regulation of energy intake in the medium and long term in the chicken, rather than on a shorter term meal to meal basis (Boswell and Dunn, 2017). Latency to reach the wood shavings platform in the motivation test decreased just before being fed indicating an increase in motivation at that point. It has previously been found that motivation increases as time since last feeding increases (e.g. Savory and Lariviere, 2000) but these tests involve the birds working for a food reward whereas our motivation test only allowed appetitive feeding behavior (foraging) and may account for the lack of change in motivation until shortly before the next feeding (see D'Eath et al., 2009 for criticisms of feeding motivation tests).

Ad Libitum Versus Restricted Diets

Feed treatment (AL vs Ram and Rpm) had a more significant impact on our measures than time since last feeding: AL birds were heavier (grew faster) and had some larger digestive organs (gall bladder, gizzard, liver, pancreas and proventriculus) compared to R treatment birds. Additionally, AL birds had lower levels of physiological indicators of hunger, such as gene expression of the orexigenic neuropeptides AGRP and NPY, higher levels of factors related to satiety, such as expression of the anorectic gene POMC in the basal hypothalamus and PYY and PPY in the pancreas (Reid et al., 2017). However, previously we did not detect any changes in POMC mRNA expression in the AL vs R fed birds but this may be due to a smaller sampler size or greater variation in the previous study (Dunn et al., 2013b). It may be that in an even larger powered study, differences in POMC expression over the 24 hours would also be observed since it was numerically highest 1-3 h from lights on and then decreased with time since feeding.

Plasma NEFA concentrations were also lower in AL birds just prior to feeding, than in restricted-fed which indicates that AL birds were able to store more energy and R treatment birds had to use more energy reserves. CCK has previously been found to inhibit food intake (Savory, 1980), and its receptors are less abundant in chickens bred for fast growth (Dunn et al., 2013a). The type of broiler breeders used in this study are the parent stock to one of the fastest growing broiler strains commercially available (Ross 308: Aviagen, 2013). However, the results suggest that although CCKAR expression may underlie growth differences, the expression of this receptor is not responsive to diet-induced changes in growth and feed intake. Additionally, similar to the results found by de Jong et al (2003), although there were no differences found in plasma glucose concentrations between ad libitum and restricted fed birds at most time points sampled after feeding the levels in the Ram group immediately after feeding were higher.

We also found no changes in CART gene expression in response to food restriction. This may reflect that we used females in our study because previous observations of decreased CART mRNA in chickens in response to food deprivation or restriction have only been observed in males (Cai et al., 2015; Caughey et al., 2018). Additionally, circulating insulin and glucagon peptide levels are positively correlated, respectively, with feed intake and fasting in chickens (Simon, 1989; Richards and McMurtry, 2008) but we did not see any differences in their gene expressions between AL birds and R birds, despite a 3-4 fold difference in feed intake. This suggests that changes in circulating insulin and glucagon are produced by post-translational effects or changes in secretion rather than by altered gene expression as indicated for glucagon by Richards and McMurtry (2008).

From the behavioral data, AL birds spent more time feeding and less time walking during the lights on period in the home pen than the restricted fed birds. AL birds also spent less time walking during the dark period than Rpm birds; although the majority of the lights off period

729 was spent inactive for all feed treatments as birds naturally sleep during darkness periods
730 (Blokhuis, 1984). Additionally, AL birds were also less motivated (less successful, higher
731 latency) to access an area with a foraging substrate than similarly aged Ram and Rpm birds.
732 These results are similar to our previous experiments (e.g. (Boswell et al., 1999, 2002; Dunn
733 et al., 2013b; a; Dixon et al., 2014; Reid et al., 2017) and others who have compared ad
734 libitum or larger portion fed broiler breeders with those that were restricted in food quantity
735 (e.g. Hocking et al., 1993; de Jong et al., 2003; Bokkers and Koene, 2004; Lees et al., 2017;
736 Arrazola et al 2020).

737 ***Feed Treatment by Time Relative to Last Meal Interactions***

738 The combination of time since last feeding and feed treatment corresponded with changes in
739 several measures. As AL birds could feed throughout the day, they had similar crop weight
740 scores with a significant peak at 7-9 h after feeding which then decreased over time, crop
741 content scores which had a slight increase over time and NEFA concentrations which had a
742 peak at 16-18 h after feeding. In contrast Ram and Rpm birds had very high crop weight and
743 content scores just after feeding, while the crop essentially becomes empty 22-24 h after
744 feeding. Our finding that both R treatment birds showed high plasma NEFA concentrations
745 just before feeding is consistent with other research that found a peak in plasma NEFA at 20-
746 24 h post feeding in restricted birds (de Beer et al., 2008), and is consistent with a
747 mobilization of body energy reserves.
748 Ram birds had a peak in plasma glucose right after being fed which decreased as time since
749 feeding increased, while AL and Rpm birds had consistent glucose levels. This may be due to
750 the slower digestion times in Rpm birds which were fed shortly before lights off and have
751 less demand for glucose due to reduced activity in the dark period.
752 In a complementary paper (Reid et al., 2017) which sought to correct mistakes in the chicken
753 genome regarding the PP fold family of peptides, we measured both PPY and PYY

754 expression. This paper adds the expression in the pancreas of the 3rd member of the family,
755 NPY but the primary surprise was that PYY is expressed highly in the pancreas of chickens,
756 something which is not an obvious feature of mammalian physiology. In the pancreas, PYY
757 is known for its roles in maintaining glucose while PPY is related to satiety, principally
758 thought to be secreted from the small intestine (Boey et al., 2007). In the Reid et al (2017)
759 study, we found that PPY was clearly different between feeding treatments and was
760 numerically but not significantly lower in the AL group during the night.
761 This finding for PPY was replicated in this paper on a larger set of the same samples. PYY
762 expression was higher in the pancreas of chickens than in other gut tissues sampled, and both
763 PPY and PYY were higher in the pancreas of AL fed birds. PYY did change with time of
764 sampling relative to feeding: PYY expression was higher 7 h after feeding in Ram and Rpm
765 birds and lower in AL fed birds at night, reaching expression levels similar to those seen in
766 both R treatment birds. In contrast in the present study NPY showed no effect of time of day
767 or treatment consistent with its role in the gut as a neurotransmitter in peripheral nerves rather
768 than as a secreted peptide. Therefore, PYY may also act as a short term satiety factor in birds
769 (Reid et al., 2017) and may show good correlation with behavioral effects on feeding
770 motivation which we aim to test further in the future.
771 In the foraging motivation test, the behavior of the Rpm birds on the platforms changed as the
772 time post feeding increased; they increased standing and the standing/sitting combined
773 measure on the start platform and walking on the wood shavings platform. Both Ram and
774 Rpm birds decreased their foraging on the start platform by 22-24 h since last feed while Ram
775 birds had a decrease in walking and increase in foraging at 7-18 h since last feed on the wood
776 shavings platform but these reversed at 22-24 h with walking increasing and foraging
777 decreasing. Broiler breeders have been shown to increase locomotor (walking) behavior
778 leading up to feeding time especially when they are food restricted (Kostal et al., 1992;

779 Savory and Maros, 1993). Ram birds did not show a similar increase in walking on the start
780 platform but the dark period (when birds are generally less active) was just before their meal
781 time, while Rpm birds were fed towards the end of the light period which may account for
782 this difference (Savory, 1980; Dixon et al., 2016).
783 For home pen behavior, the time of day had a larger effect than time since last feeding.
784 During the light period, AL birds increased their feeding throughout the light period but Ram
785 and Rpm birds decreased their feeding and pecking at the feeder, most likely because the
786 feeders got emptied quickly. AL and Ram birds also foraged and walked regularly throughout
787 the light period while Rpm birds decreased foraging and increased walking as it got closer to
788 their feeding time, showing the pre-feeding increase in locomotor behavior mentioned above
789 and found in other studies (reviews in Mason and Mendl 1997; D'Eath et al., 2009). AL and
790 Ram birds drank uniformly throughout the light period while the Rpm birds decreased their
791 drinking. Restricted broiler breeders often display polydipsia as an attempt to gut fill and
792 commercial breeders are often water restricted to prevent this (Savory et al., 1992). It is
793 possible the Rpm birds drank enough to achieve gut fill earlier in the day and therefore did
794 not need to continue at high drinking levels, or they may have reduced drinking to 'leave
795 room' for their expected afternoon meal.
796 During the dark period, AL and Ram birds were most active before lights on with mainly
797 walking and preening behavior, possibly in anticipation of their upcoming feeding
798 (Mistlberger and Rusak, 1987; Wichman et al., 2012) whereas Rpm birds were most active
799 after lights off (shortly after they were fed), again mainly with walking and preening
800 behavior, but their activity levels were still similar to AL birds before lights on.
801 ***Foraging Test Success***
802 A typical design of motivation tests is to increase the cost of accessing the resource over
803 subsequent tests, which was done here as an increase in the length and depth of the water

804 runway over 4 tests. Animals who are highly motivated to access a resource should continue
805 to work for it, while those not motivated should stop responding (Dawkins, 1990). The
806 proportion of birds reaching the wood shavings platform (successful birds) did decrease and
807 the latency to the wood shavings platform did increase in tests 3 and 4. However, the success
808 rate was only 25% at its highest and decreased to 9% at its lowest. These numbers are low
809 because of the inclusion of AL birds in the analysis, who were rarely successful in
810 completing the motivation test. Only 0.4% of AL birds were successful over the 4 tests
811 combined while 62% Ram birds and 57% Rpm birds succeeded in reaching the wood
812 shavings platform. AL birds always had access to feed so would not be expected to be
813 motivated to reach an area where they can search for more food, especially given the increase
814 in cost to reach that area over the 4 tests and this is similar to previous results (Dixon et al.,
815 2014).
816 When examining the R treatments only, test success ranged from 46-69% for Ram birds and
817 44-59% for Rpm birds. These values are a little lower than those found in previous work,
818 where we found a success rate of over 90% in R birds for the tests with the easier costs,
819 reducing to over 60% success in the hardest test (Dixon et al., 2014). The main difference
820 between the current study and Dixon et al (Dixon et al., 2014) was the training and testing of
821 birds. Previously birds were given 10 minutes to reach the wood shavings platform and if
822 they were successful, they were then allowed 5 more minutes to spend on the wood shavings
823 platform (although birds could leave the wood shavings platform before the 5 minutes were
824 up if they chose to). In this experiment, the test was ~20 minutes in total and the birds could
825 spend this time in any area of the apparatus that they chose. This means the birds had more
826 time to visit the wood shavings platform and this may have led to more rapid learning that
827 there is no food in the foraging area, which would de-value the reward (Apps et al., 2015).
828 Successfully reaching the wood shavings platform was never rewarded with feed so it may be

829 expected that the responses might extinguish (Bouton, 2004). However, a large proportion of
830 the Ram and Rpm birds continued to work for access to the wood shavings platform even as
831 the cost increased indicating that they were still motivated to search for food (Stephens and
832 Krebs, 1986).

833 In conclusion, there were changes to several behavioral and physiological measures
834 throughout the 24-hour period. However, there are time windows where future data can be
835 collected where changes due to time of day and/or time since last feeding will not have a
836 major influence on findings. Additionally, this experiment provides further evidence that feed
837 restricted birds show behavioral and physiological signs of hunger and that the amount of
838 feed provided has the largest effect on most of these measures compared to any other feeding
839 driven or diurnal rhythms produced by feeding time. In terms of hunger/satiety regulation, it
840 appears that AGRP, NPY (basal hypothalamus), POMC and plasma NEFA are most sensitive
841 to feeding history in fast growing chickens than other potential physiological indicators.
842 From an animal welfare perspective, restricted feeding of broiler breeders is still a concern
843 that needs to be addressed. In subsequent studies we have used these measures to investigate
844 the feeding of broiler breeders with adjusted diets to try and improve satiety and therefore
845 welfare. For example, increased dietary fibre and/or lower energy and protein diets has been
846 investigated. If a feeding solution to feed restriction in broiler breeders can be found, it has
847 the potential to improve the welfare of millions of birds in the UK and worldwide.

848

849 **ACKNOWLEDGEMENTS**

850 The work described in the present study was funded by BBSRC grant BB/L000288/1
851 'Investigating how the type and quantity of food affect foraging behavior and the neural
852 circuits controlling feeding in broiler breeder chickens'. SRUC and BioSS receive funding
853 from the Scottish Government's Environment, Agriculture and Food Strategic Research

Programme and the Roslin Institute is funded by the BBSRC through Institute Strategic Grant funding BB/J004316/1. The authors would like to thank the staff at the Monogastric Science Research Centre for providing excellent animal care and the manuscript reviewers for their helpful feedback.

Declarations of interest/Conflicts of Interest: None

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Table 1: Effects of the feed treatments on physiological measurements. Values are means and SEMs estimated from LMMs. If the data were analysed on transformed scale these values are shown, with back-transformed values shown in brackets where biologically meaningful.

	Feed Treatment				Statistics	
Physiological Measures	**AL**	**Ram**	**Rpm**	**SEM**	**F or Wald†**	**P**
Weight at PM (g)	8.012a (3016)	7.083b (1191)	7.098b (1210)	0.015	1234.32	<0.001
Plasma NEFA	-2.45a (0.086)	-2.09b (0.124)	-1.88b (0.152)	0.14	16.31	<0.001
Plasma glucose	2.409 (11.1)	2.410 (11.1)	2.377 (10.8)	0.043	3.05	ns
AGRP (bh)	-6.71a	-3.67b	-3.61b	0.35	*252.59*	<0.001
NPY (bh)	-4.00a	-3.09b	-2.97b	0.10	84.82	<0.001
POMC (bh)	-4.29b	-5.11a	-5.19a	0.28	26.17	<0.001
CART (bh)	-4.01	-4.09	-3.98	0.27	1.55	ns
CCKAR (pan)	-0.67	-0.42	-0.46	0.12	1.57	ns
GCG (pan)	-2.31	-1.67	-2.48	0.35	2.31	ns
insulin (pan)	2.46	2.29	2.13	0.14	1.32	ns
NPY (pan)	-5.60	-5.43	-5.38	0.10	2.25	ns
PPY (pan)	3.82a	3.08b	2.98b	0.19	10.74	0.002
Gall bladder (empty) (g)	-0.848c (0.428)	-1.586a (0.205)	-1.462b (0.232)	0.044	80.94	<0.001
Gizzard (g)	4.173a (64.9)	3.970b (53.0)	3.988b (54.0)	0.048	12.81	0.001
Liver (g)	4.18a (65.3)	3.031b (20.7)	3.11b (22.5)	0.042	399.62	<0.001
Pancreas (g)	1.812a (6.12)	1.056b (2.88)	1.040b (2.83)	0.023	440.99	<0.001
Proventriculus (g)	2.371c (10.7)	1.599b (4.95)	1.542a (4.67)	0.021	618.84	<0.001
Crop content weight (g)	2.87 (16.7)	2.43 (10.4)	2.58 (12.2)	0.16	2.23	ns
Crop content Score (1-5)	2.84a	2.52b	2.56b	0.21	*6.83*	0.033

bh = measured from the basal hypothalamus

pan = measured from the pancreas

ns = non-significant (P>0.05)

SEM = highest standard error of the mean for each factor

Superscripted letters indicate where differences lie

Treatments sharing a letter do not differ significantly from each other

† ndf=2, ddf=13-181, italic text indicates Wald tests used

Table 2: Effects of the feed treatments on the foraging motivation test measurements. Values are means and SEMs estimated from LMMs or GLMMs. If the data were analysed on transformed scale these values are shown, with back-transformed values shown in brackets where biologically meaningful.

	Feed Treatment				Statistics	
Foraging Motivation Test Measurements	**AL**	**Ram**	**Rpm**	**SEM**	**F or Wald†**	**P**
Foraging test success (proportion of birds) (GLMM)§	5.46[a] (0.004)	0.51[b] (0.624)	0.27[b] (0.567)	0.84	*42.35*	<0.001
R birds: Foraging test success (proportion of birds) (GLMM)	NA	0.59 (0.644)	0.29 (0.572)	0.43	*0.22*	ns
Latency to wood shavings platform (s)	89.1[a] (1200 s)	55.2[b] (809 s)	53.8[b] (781 s)	3.0	*97.12*	<0.001
R birds: Latency to wood shavings platform (s)	NA	55.2 (809 s)	53.8 (781 s)	3.5	*0.03*	ns
Proportion of test spent on start platform	78.6[a] (0.961)	44.6[b] (0.492)	44.9[b] (0.498)	2.8	*126.26*	<0.001
Proportion of test spent on wood shavings platform	0.5[b] (0.000)	19.3[a] (0.109)	18.6[a] (0.102)	1.9	*74.22*	<0.001
R birds: Proportion of test spent on wood shavings platform	NA	19.3 (0.109)	18.6 (0.102)	2.2	*0.41*	ns
Proportion of time on the start platform spent standing/sitting (test numbers 1 and 4)	71.9[a] (0.904)	49.4[b] (0.576)	51.0[b] (0.604)	1.7	73.94	<0.001
Proportion of time on the start platform spent standing (test numbers 1 and 4)	44.8[b] (0.497)	49.4[ab] (0.577)	51.0[a] (0.603)	1.8	6.18	0.009
Proportion of time on the start platform spent preening (test numbers 1 and 4)	5.1[c] (0.0078)	13.3[a] (0.0528)	8.8[b] (0.0235)	2.3	11.72	<0.001
Proportion of time on the start platform spent foraging (test numbers 1 and 4)	14.2[b] (0.061)	26.9[a] (0.204)	26.6[a] (0.200)	1.6	24.75	<0.001
Proportion of time on the start platform spent walking (test numbers 1 and 4)	3.54[c] (0.0038)	11.15[b] (0.0374)	13.66[a] (0.0558)	0.91	37.93	<0.001
Successful R birds: Proportion of time on the wood shavings platform spent standing	NA	16.1 (0.0767)	16.2 (0.078)	2.0	*0.29*	ns
Successful R birds: Proportion of time on the wood shavings platform spent preening‡	NA	9.5[a] (0.0270)	4.8[b] (0.0071)	1.4	*4.06*	0.044
Successful R birds: Proportion of time on the wood shavings platform spent foraging	NA	60.7 (0.760)	65.7 (0.831)	2.6	*0.14*	ns
Successful R birds: Proportion of time on the wood shavings platform spent walking	NA	9.4 (0.0265)	10.1 (0.0308)	1.7	*2.32*	ns

ns = non-significant (P>0.05)

SEM = highest standard error of the mean for each factor

Superscripted letters indicate where differences lie

Treatments sharing a letter do not differ significantly from each other

† ndf=2 or 1 for R birds only, ddf=18-183, italic text indicates Wald tests used

§ only main fixed effects included

ǂ only 2 way interaction and main fixed effects included

1093 Table 3: Effects of the feed treatments on the home pen behaviour measurements. Values are means and SEMs estimated from GLMMs. If the
1094 data were analysed on transformed scale these values are shown, with back-transformed values shown in brackets where biologically
1095 meaningful.

	Feed Treatment				Statistics	
Lights ON	**AL**	**Ram**	**Rpm**	**SEM**	**F or Wald†**	**P**
Proportion of time spent feeding‡	-2.32^{a} (0.0891)	-3.15^{b} (0.0411)	-3.53^{b} (0.0285)	0.19	11.69	<0.001
Proportion of time spent foraging	-1.94 (0.125)	-2.02 (0.118)	-1.76 (0.147)	0.36	1.01	ns
Proportion of time spent drinking	-2.33^{b} (0.089)	-1.16^{a} (0.239)	-2.08^{b} (0.111)	0.15	17.46	<0.001
Proportion of time spent object pecking	-3.59^{b} (0.0268)	-1.97^{a} (0.1224)	-3.24^{b} (0.0376)	0.20	18.44	<0.001
Proportion of time spent preening	-1.89^{a} (0.1315)	-2.92^{c} (0.0513)	-2.54^{b} (0.0729)	0.12	31.93	<0.001
Proportion of time spent walking	-2.65^{b} (0.0657)	-2.10^{a} (0.1091)	-2.09^{a} (0.1103)	0.14	21.14	<0.001
Proportion of time spent being active (walking + foraging)	-1.39^{b} (0.200)	-1.14^{ab} (0.242)	-0.90^{a} (0.289)	0.32	4.70	0.021
Proportion of time spent being inactive (standing, sitting, sleeping)	-0.63^{a} (0.347)	-2.17^{b} (0.103)	-0.71^{a} (0.330)	0.22	40.51	<0.001
Lights OFF	**AL**	**Ram**	**Rpm**	**SEM**	**F or WaldŦ**	**P**
Proportion of time spent feeding§	-4.34^{a} (0.01289)	-6.41^{b} (0.00164)	-4.62^{a} (0.00972)	0.42	*20.47*	<0.001
Proportion of time spent foraging§	-6.59^{b} (0.00137)	-7.69^{b} (0.00046)	-5.00^{a} (0.00671)	0.80	5.97	0.006
Proportion of time spent drinking§	-4.46^{b} (0.0114)	-5.41^{c} (0.0044)	-3.18^{a} (0.0398)	0.30	24.04	<0.001
Proportion of time spent object pecking§	-6.92^{b} (0.000986)	-8.06^{b} (0.000316)	-5.79^{a} (0.003042)	0.76	*12.33*	0.002
Proportion of time spent preening	-3.20 (0.0392)	-3.31 (0.0352)	-3.53 (0.0285)	0.13	2.62	ns
Proportion of time spent walking‡	-4.41^{b} (0.0121)	-4.00^{b} (0.0180)	-3.34^{a} (0.0341)	0.20	9.30	0.001
Proportion of time spent being active (walking + foraging)†	-4.20^{b} (0.0147)	-3.91^{b} (0.0196)	-3.03^{a} (0.0461)	0.19	12.47	<0.001
Proportion of time spent being inactive (standing, sitting, sleeping)	2.35^{b} (0.913)	2.73^{a} (0.939)	1.78^{c} (0.855)	0.11	19.67	<0.001

ns = non-significant (P>0.05)

SEM = highest standard error of the mean for each factor

Superscripted letters indicate where differences lie

Treatments sharing a letter do not differ significantly from each other

† ndf=2, ddf=19-290

Ŧ ndf=2, ddf=22-129

Italic text indicates Wald tests used

ǂ only 2 way interaction and main fixed effects included

§ only main fixed effects included

1108 Table 4: Effects of time since last feed on physiological measurements. Values are means and
1109 SEMs estimated from LMMs. If the data were analysed on transformed scale these values are
1110 shown, with back-transformed values shown in brackets where biologically meaningful.

	Time since last feed					Statistics	
Physiological Measures	**1-3**	**7-9**	**16-18**	**22-24**	**SEM**	**F or WaldT**	**P**
Weight at PM (g)	7.375[b] (1595)	7.431[a] (1688)	7.413[ab] (1658)	7.371[b] (1590)	0.018	2.70	0.047
Plasma NEFA	-2.32[b] (0.098)	-2.75[a] (0.064)	-2.04[c] (0.130)	-1.45[d] (0.234)	0.15	41.21	<0.001
Plasma glucose	2.465[a] (11.8)	2.398[b] (11.0)	2.354[c] (10.5)	2.377[bc] (10.8)	0.043	13.10	<0.001
AGRP (bh)	-4.23[b]	-4.86[a]	-4.82[a]	-4.74[a]	0.36	*9.10*	0.028
NPY (bh)	-3.24	-3.38	-3.44	-3.35	0.11	1.95	ns
POMC (bh)	-4.79	-4.86	-4.93	-4.87	0.28	0.29	ns
CART (bh)	-4.07	-3.97	-4.04	-4.03	0.28	0.49	ns
CCKAR (pan)	-0.45	-0.52	-0.47	-0.64	0.14	0.37	ns
GCG (pan)	-2.09	-1.94	-2.69	-1.89	0.39	1.15	ns
insulin (pan)	2.44	2.40	2.26	2.08	0.16	1.04	ns
NPY (pan)	-5.49	-5.50	-5.49	-5.39	0.11	0.30	ns
PPY (pan)	3.42	3.37	3.21	3.17	0.20	0.70	ns
Gall bladder (empty) (g)	-1.330[a] (0.264)	-1.377[a] (0.252)	-1.327[a] (0.265)	-1.159[b] (0.314)	0.050	3.97	0.009
Gizzard (g)	4.080[ab] (59.1)	4.096[a] (60.1)	4.016[bc] (55.5)	3.983[c] (53.7)	0.046	3.77	0.012
Liver (g)	3.359[c] (28.8)	3.528[a] (34.1)	3.476[ac] (32.3)	3.401[bc] (30.0)	0.041	7.07	<0.001
Pancreas (g)	1.287 (3.62)	1.325 (3.76)	1.293 (3.65)	1.307 (3.70)	0.026	0.45	ns
Proventriculus (g)	1.838 (6.29)	1.849 (6.35)	1.815 (6.14)	1.846 (6.33)	0.024	0.45	ns
Crop content weight (g)	3.79[a] (43.2)	3.46[a] (30.9)	2.36[b] (9.5)	0.90[c] (1.5)	0.14	128.79	<0.001
Crop content Score (1-5)	3.50[a]	2.80[b]	2.44[b]	1.81[c]	0.22	*98.33*	<0.001

1111 bh = measured from the basal hypothalamus

1112 pan = measured from the pancreas

1113 ns = non-significant (P>0.05)

1114 SEM = highest standard error of the mean for each factor

1115 Superscripted letters indicate where differences lie

1116 Treatments sharing a letter do not differ significantly from each other

1117 Ŧ ndf=3, ddf=147-181, italic text indicates Wald tests used

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1131 Table 5: Effects of the time since last feed on the foraging motivation test measurements. Values are means and SEMs estimated from LMMs or
1132 GLMMs. If the data were analysed on transformed scale these values are shown, with back-transformed values shown in brackets where
1133 biologically meaningful.

	Time since last feed				Statistics	
Foraging Motivation Test Measurements	**1-3**	**7-18**	**22-24**	**SEM**	**F or WaldF**	**P**
Foraging test success (proportion of birds) (GLMM)[§]	-1.91 (0.129)	-1.96 (0.124)	-0.82 (0.305)	0.50	*4.72*	ns
R birds: Foraging test success (proportion of birds) (GLMM)	-0.14 (0.465)	-0.01 (0.499)	1.47 (0.813)	0.58	*4.58*	ns
Latency to wood shavings platform (s)	69.1^{a} (1048 s)	69.4^{a} (1051 s)	59.6^{b} (893 s)	3.3	*7.13*	0.028
R birds: Latency to wood shavings platform (s)	59.8^{a} (896 s)	59.3^{a} (887 s)	44.4^{b} (587 s)	4.7	*7.69*	0.021
Proportion of test spent on start platform	55.8 (0.684)	58.3 (0.724)	54.0 (0.654)	3.1	*1.56*	ns
Proportion of test spent on wood shavings platform	10.9 (0.036)	12.3 (0.046)	15.2 (0.068)	2.1	*2.57*	ns
R birds: Proportion of test spent on wood shavings platform	15.6 (0.072)	18.5 (0.101)	22.8 (0.15)	3.0	*2.94*	ns
Proportion of time on the start platform spent standing/sitting (test numbers 1 and 4)	54.2^{b} (0.657)	59.5^{a} (0.743)	58.6^{ab} (0.729)	1.8	3.67	0.027
Proportion of time on the start platform spent standing (test numbers 1 and 4)	48.6 (0.562)	48.0 (0.551)	48.7 (0.564)	2.0	0.05	ns
Proportion of time on the start platform spent preening (test numbers 1 and 4)	8.2^{b} (0.0201)	7.1^{b} (0.0152)	11.9^{a} (0.0428)	2.4	4.54	0.012
Proportion of time on the start platform spent foraging (test numbers 1 and 4)	27.1^{a} (0.207)	22.9^{a} (0.151)	17.7^{b} (0.093)	1.8	7.27	<0.001
Proportion of time on the start platform spent walking (test numbers 1 and 4)	8.82^{b} (0.0235)	8.00^{b} (0.0194)	11.53^{a} (0.0399)	0.98	4.45	0.013
Successful R birds: Proportion of time on the wood shavings platform spent standing	16.5^{ab} (0.0809)	12.5^{b} (0.0471)	19.4^{a} (0.1102)	2.8	*9.12*	0.010
Successful R birds: Proportion of time on the wood shavings platform spent preening[ł]	6.1 (0.0113)	7.6 (0.0173)	7.8 (0.0183)	2.0	*0.46*	ns
Successful R birds: Proportion of time on the wood shavings platform spent foraging	61.8^{ab} (0.777)	68.2^{a} (0.862)	59.6^{b} (0.744)	3.8	*7.80*	0.020
Successful R birds: Proportion of time on the wood shavings platform spent walking	10.0^{ab} (0.0302)	6.6^{b} (0.0133)	12.6^{a} (0.0474)	2.3	*8.82*	0.012

1134 ns = non-significant (P>0.05)

1135 SEM = highest standard error of the mean for each factor

1136 Superscripted letters indicate where differences lie

1137 Treatments sharing a letter do not differ significantly from each other

1138 Ŧ ndf=2, ddf=167-183, italic text indicates Wald tests used

1139 § only main fixed effects included

1140 ǂ only 2 way interaction and main fixed effects included

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1149 Table 6: Effects of the time in the day on the home pen behaviour measurements. Values are means and SEMs estimated from GLMMs. If the
1150 data were analysed on transformed scale these values are shown, with back-transformed values shown in brackets where biologically
1151 meaningful.

	Time in the day/night				Statistics	
Lights ON	**8:00-10:30**	**10:30-13:30**	**13:30-16:00**	**SEM**	**F or Wald‡**	**P**
Proportion of time spent feeding[ǂ]	-2.78 (0.0582)	-3.20 (0.0391)	-3.02 (0.0467)	0.16	1.49	ns
Proportion of time spent foraging	-1.79[a] (0.143)	-1.87[ab] (0.134)	-2.06[b] (0.113)	0.35	3.25	0.042
Proportion of time spent drinking	-1.49[a] (0.184)	-1.89[b] (0.131)	-2.19[c] (0.101)	0.10	38.90	<0.001
Proportion of time spent object pecking	-2.61[a] (0.0686)	-3.05[b] (0.0451)	-3.14[b] (0.0414)	0.14	8.17	<0.001
Proportion of time spent preening	-2.54[b] (0.0732)	-2.23[a] (0.0975)	-2.58[b] (0.0703)	0.10	9.03	<0.001
Proportion of time spent walking	-2.38[b] (0.0845)	-2.44[b] (0.0799)	-2.02[a] (0.1175)	0.14	27.94	<0.001
Proportion of time spent being active (walking + foraging)	-1.12 (0.246)	-1.23 (0.227)	-1.09 (0.252)	0.31	1.56	ns
Proportion of time spent being inactive (standing, sitting, sleeping)	-1.76[b] (0.147)	-0.93[a] (0.282)	-0.82[a] (0.306)	0.20	62.19	<0.001
Lights OFF	**16:30-20:00**	**22:30-01:45**	**4:30-07:45**	**SEM**	**F or Wald¥**	**P**
Proportion of time spent feeding[§]	-5.00 (0.00671)	-5.49 (0.00410)	-4.89 (0.00750)	0.26	*5.08*	ns
Proportion of time spent foraging[§]	-5.88 (0.00278)	-7.10 (0.00083)	-6.29 (0.00185)	0.52	2.87	ns
Proportion of time spent drinking[§]	-4.26[a] (0.0140)	-4.82[b] (0.0080)	-3.98[a] (0.0184)	0.21	6.13	0.003
Proportion of time spent object pecking[§]	-6.16[a] (0.002106)	-7.40[b] (0.000612)	-7.21[b] (0.000737)	0.55	*8.48*	0.014
Proportion of time spent preening	-3.57[b] (0.0275)	-3.84[b] (0.0210)	-2.64[a] (0.0668)	0.14	33.33	<0.001
Proportion of time spent walking[ǂ]	-4.08[b] (0.0167)	-4.28[b] (0.0136)	-3.39[a] (0.0327)	0.19	10.31	<0.001
Proportion of time spent being active (walking + foraging)[ǂ]	-3.79[b] (0.0222)	-4.12[b] (0.0160)	-3.24[a] (0.0376)	0.17	11.07	<0.001
Proportion of time spent being inactive (standing, sitting, sleeping)	2.42[b] (0.918)	2.76[a] (0.941)	1.68[c] (0.842)	0.10	29.94	<0.001

ns = non-significant (P>0.05)

SEM = highest standard error of the mean for each factor

Superscripted letters indicate where differences lie

Treatments sharing a letter do not differ significantly from each other

‡ ndf=2, ddf=104-156

¥ ndf=2, ddf=83-209

Italic text indicates Wald tests used

ǂ only 2 way interaction and main fixed effects included

§ only main fixed effects include

1161 Figure Titles

1162 Fig 1: The allocation of birds to pens, treatments and rooms for both batches of the
1163 experiment.

1164 Fig 2. The treatment structure for the experiment, showing time relative to feeding and actual
1165 time of day when measurements took place for the 3 feed treatments. Birds were culled for
1166 PMs during ~2 hour intervals starting at 1, 7, 16 and 22 hours relative to feeding. Observation
1167 times for AL were chosen to match those for Ram. These time intervals were chosen in order
1168 to have 1 soon after feeding, 1 just before feeding, and 2 intermediate, and so that 3 out of 4
1169 intervals also coincided at the same times in the day.
1170 Foraging tests took place for each bird at the same time in the day that the bird was to be
1171 culled for post mortem, apart from those culled around midnight for which foraging tests
1172 were instead at 17:00-19:00 (Ram) or 5:00-7:00 (Rpm). (Foraging motivation tests were not
1173 carried out at midnight as the birds would have been asleep for a few hours and previous
1174 experience suggests they would not perform in the motivation test). Home pen scan sessions
1175 were chosen to also coincide with the time in the day birds were culled for post mortem, plus
1176 the addition of 1 session in the middle of the day. Each 1-hour session contained 10 scans
1177 during lights on and 5 scans during lights off. Foraging tests took place over 3 weeks per
1178 batch with different birds being tested each week, and then home pen observations took place
1179 for all birds over 1 24-hour period at the end of each of these weeks, when birds were
1180 undisturbed, apart from for feeding.

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1182 Fig 3: Back-transformed crop content weight (a), crop content score (b), plasma NEFA levels
1183 (c) and plasma glucose levels (d) for each feed treatment at the 4 sampling times relative to
1184 last feed (hours). Data are back-transformed means±SEMs estimated from LMMs.

Fig 4: Back-transformed means and SEM for the proportion of the test time spent standing/sitting (a), standing only (b) and foraging (c) on the start platform and for the proportion of the test time spent foraging (d) and walking (e) for the successful R birds on the wood shavings platform at the 3 sampling times relative to last feed (hours). Data are back-transformed means±SEMs estimated from LMMs.

Fig 5: Back-transformed means of the time spent feeding (a), foraging (b), drinking (c), preening (d), walking (e) and inactive (f) during the lights on period in the home pen. Data are back-transformed means±SEMs estimated from GLMMs.

Fig 6: Back-transformed means of the time spent preening (a), walking (b), active (c) and inactive (d) during the lights off period in the home pen. Data are back-transformed means±SEMs estimated from GLMMs.

Fig 7: Back-transformed means of the latency to reach the wood shaving platform (a), the proportion of the test time spent on the start platform (b) and the proportion of the test time spent on the wood shavings platform (c) over the 4 tests and the proportion of the test time spent preening (d) and walking (e) on the start platform over tests 1 and 4. Data are back-transformed means±SEMs from LMMs.