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Dilution of broiler breeder diets with oat hulls prolongs feeding but does not affect central control of appetite

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ABSTRACT The parents of broiler (meat) chickens (ie, broiler breeders) are food-restricted until sexual maturity, ensuring good health and reproduction, but resulting in hunger. We investigated whether diets with added insoluble fiber promote satiety and reduce behavioral, motivational, and physiological signs of hunger. Ninety-six broiler breeders were fed 1 of 4 feed treatments ($n = 24$ per diet) from 6 to 12 wk of age: 1) a commercial diet fed to the recommended ration (R) or 2) ad libitum (**AL**), the same diet as R but mixed with oat hulls at 3) 20% (OH20%) or 4) 40% (OH40%). The R, OH20% and OH40% diets were approximately iso-energetic and resulted in mean 12 wk of age weights within 2.5% of each other (1.21 kg), while AL birds weighed 221% as much (2.67kg). At 12 wk of age, agouti-related protein (***AGRP***) expression, was, on average, more than 12 times lower in AL birds ($P < 0.001$) but did not differ between the fiber diet treatments and R. Pro-opiomelanocortin (***POMC***) expression, was, on average, over 1.5 times higher in AL birds, but was not statistically significantly affected by feed treatments ($P = 0.33$). In their home pens, AL birds stood/sat more, foraged less and fed more in total ($P < 0.001$) and OH40% birds spent longer feeding than R ($P = 0.001$). Motivation to forage tested by willingness to walk through water to access an area of wood shavings (without food) was not significantly affected by diet ($P = 0.33$). However, restricted birds were willing to cross in only 7.3% to 12.5% of tests. Mostly birds stayed on the start platform, where AL birds sat more than other treatments and OH40% birds reduced walking relative to R birds ($P = 0.016$). Across the behavioral and physiological measurements there was a dichotomy of effects in response to approximately iso-energetic diets differing in fiber. There were some potentially beneficial behavioral effects related to reduced foraging and walking. However, there was no evidence that these diets significantly improved physiological measures of satiety of broiler breeders.

Key words: broiler breeder, feed restriction, behavior, motivation, *AGRP*

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INTRODUCTION

Genetic selection for rapid growth and feed efficiency in broiler (meat) chickens has resulted in 3-fold increases in these traits over a 50-y period (Havenstein et al., 2003), with commercially used "fast growing" breeds now reaching their 2.5 kg slaughter weight as juveniles at 42 d or less. Their parents (broiler breeders) reach sexual maturity at around 20 wk of age and are typically used to produce eggs until 60+ wk (EFSA-AHAW, 2010). Ad libitum feeding of these birds results in obesity and poor welfare (lameness, thermal discomfort), reduced productivity (poor shell quality, multiple ovulations), increased morbidity and mortality of up to 31% (Mench, 2002; Heck et al., 2004; de Jong and Guemene, 2011). To avoid these problems, feed is rationed, to as little as 45% of ad libitum intake (when compared to an ad libitum-fed bird of a similar weight) for parts of the rearing period (wk 7−14). Although broiler breeders do not appear to suffer physical ill effects of hunger, they do have a very strong desire to eat and show behavioral, motivational and stress indicators related to the inability to do so which results in welfare concerns over hunger (Hocking et al., 1993; Savory et al., 1993; Dunn et al., 2013). During rearing, broiler breeders consume their

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daily ration in under 10 min, after which they show a number of behavioral signs of hunger (Mench, 2002; D'Eath et al., 2009; de Jong and Guemene, 2011). Locomotor and oral activity (foraging) increases, and pecks are directed towards drinkers and litter (Hocking and Jones, 2006), and stereotypic behavior linked to frustration can develop (Savory et al., 1993). Aggressive behavior (often related to food competition) can increase while comfort behaviors (such as self-preening, dustbathing), which may be indicators of positive welfare (Boissy et al., 2007), are reduced (de Jong et al., 2003). Therefore, food restriction and ad libitum feeding both result in welfare problems for broiler breeders, posing an ethical dilemma (Savory et al., 1993; Kasanen et al., 2010) referred to as the "broiler breeder paradox" (Decuypere et al., 2006).

Satiation is defined as the internal physiological processes that result in an animal choosing to end a meal, while satiety is defined as the internal physiological processes that result in the interval between meals (Forbes, 1995), or in other words, feelings of fullness and suppression of hunger for a period of time after a meal. Various researchers have examined whether alternative diets could increase satiation and satiety while restricting growth (reviewed by D'Eath et al., 2009; van Krimpen and De Jong, 2014; Nicol, 2015). Consuming the same overall energy at a reduced energy density ("qualitative" restriction) results in an increased volume of feed which almost by definition will enhance satiation because it takes longer to eat, and must be consumed over more than 1 feeding bout (meal), and may also promote satiety through increased gut fill and digestion time after a meal.

Standard broiler breeder feed can range in energy density from about 10.00 to 12.50 MJ/kg depending on breed and production phase (e.g., Aviagen, 2016; Hubbard, 2019). Feeding lower energy density feed (e.g., 9.25 MJ/kg; oat hulls at 20%, Nielsen et al., 2011; low fat, high fiber, de Jong et al., 2005), results in signs of improved welfare in some studies: comfort behaviors (Nielsen et al., 2011) and resting increase, while activity reduces (de Jong et al., 2005; Sandilands et al., 2005; Riber and Tahamtani, 2020a; but see Savory and Lariviere, 2000), although these changes are small when compared to ad libitum feeding. Behavior with high fiber feeds also becomes more "normal": feeding duration increases and abnormal behavior, such as object pecking and pacing, reduces, (Savory et al., 1996). Birds given feeds higher in fiber also have heavier gut weights and increased gut volume compared to those fed standard diets (Hetland and Svihus, 2001). These diets tend to have higher water-holding capacities and this, combined with the increased fill of the gastrointestinal tract, may increase feelings of satiety (Sykes, 1983; Whittaker et al., 1999; Hocking et al., 2004). However, some authors maintain that if energy restriction remains the same, then "metabolic hunger" will remain (Savory and Lariviere, 2000) but others dispute this view (Illius et al., 2002) and the welfare benefits of qualitative restriction remain controversial (Sandilands et al., 2005, 2006; D'Eath et al., 2009; de Jong and Guemene, 2011; Nicol, 2015).

There are also changes in markers of nutritional state in broiler breeders' response to feed restriction: plasma levels of non-esterified fatty acids (**NEFA**) decreased with increasing feed restriction but levels of plasma glucose remained similar, resulting in an increased glucose/NEFA ratio 4 to 6 h after feeding in more restricted hens (de Jong et al., 2003). When quantitative restriction was compared to qualitative restriction, the plasma glucose/NEFA ratio was higher in birds fed qualitatively restriction compared to quantitatively restricted control birds. This was the opposite of the expected result if lower glucose/NEFA indicates satiety and qualitative restriction promotes satiety. However, the authors themselves point out that without a 24-h profile of changes in the glucose/NEFA ratio, the comparisons between the diets was not valid (de Jong et al., 2005), and as plasma NEFA levels have been shown to reach a nadir at 7 to 9 h and have a peak at 22 to 24 h since last feed with levels at 1 to 3 and 16 to 18 h being intermediate (Dixon et al., 2022) sampling times in relation to meal times can greatly affect the results.

Neuroendocrine systems can also give insight into the state of hunger/satiety of broiler breeders. We investigated the effect of diet on the key components of one of the brain's food intake control centers (Boswell and Dunn, 2017) − a network of neurons in the arcuate nucleus of the hypothalamus (also known in birds as the infundibular nucleus). Here the balance of 2 pathways controls feed intake: 1) Orexigenic agouti-related protein (**AGRP**) neurons which also co-express another orexigenic peptide, neuropeptide Y (**NPY**). 2) Anorectic pro-opiomelanocortin (**POMC**) neurons, which produce α-melanocyte-stimulating hormone (**αMSH**) peptide and cocaine and amphetamine related transcript (**CART**). POMC agonises melanocortin 4 receptors (**MC4R**) within the hypothalamic paraventricular nucleus to inhibit food intake and increase energy expenditure. In contrast, AGRP acts as an antagonist at the same receptors, driving feeding behavior and promoting energy storage. In our previous studies in the broiler chicken, AGRP expression was increased but POMC expression was either unchanged or showed a small decrease in response to food restriction (Dunn et al., 2013; Caughey et al., 2018; Dixon et al., 2022), suggesting this pathway might have an important integrating role. However, these physiological indicators that vary with restriction levels or diet composition may just be measuring nutritional or metabolic state and not actually reflecting an animal's emotional state and welfare.

Animal welfare is about animals' subjective mental experiences (emotions and moods) which must be indirectly inferred (Dawkins, 2008). Here, we observed the effect of alternative diets on home pen behavior to identify behavioral changes associated with hunger/satiety such as activity, object pecking, foraging behavior and comfort behaviors, and with the potential associated impacts on welfare. We also made use of a previously developed motivational test (Dixon et al 2014) where the appetitive component of feeding (foraging) was measured using a natural cost (walking through water that can be made deeper and longer) to measure the motivation to access an area of wood shavings where appetitive (but not consummatory) feeding behaviors are possible (Dixon et al., 2014). This motivation test overcomes the issue of providing extra food during the test which may in itself increase motivation for food (the "out of sight, out of mind question," Warburton and Mason, 2003).

In this study, we reared broiler breeders to 12 wk of age adding high-fiber oat hulls at 20% and 40% inclusion rates, compared to conventional feed restriction. These 3 diet treatments provided the same amount of the basal diet which was based on the amount of feed needed to achieve the industry recommended growth target, but the quantity of the ration was increased by increasing the amount of fiber for the OH20% and OH40% treatments. Ad libitum-fed birds were also included as a positive control. To determine if diets improved satiety, we used a foraging motivation test (Dixon et al., 2014) and 2 key neuroendocrine measures from the arcuate nucleus food intake control center (gene expression of orexigenic *AGRP* and anorectic *POMC*). In addition, data on home pen behavior, metabolic measures (blood glucose and NEFA), and organ weights were collected. We hypothesized that birds fed diets with increased fiber would behave more like ad libitum fed birds but that physiological indicators of hunger would remain similar to commercially restricted fed birds.

METHODS

Animal and Housing

Ninety-six non-beak trimmed Ross 308 broiler breeder chickens, 95 females and 1 mis-sexed male (who was excluded from statistical analysis), were received from Aviagen (Stratford, UK) as day old chicks. They were housed over 2 rooms with 12 pens per room in groups of 4 birds in floor pens (1.0 × 1.5 m) covered in wood shavings. The lighting schedule for the first day was 23.5L:0.5D hours light:dark, which was then gradually altered to 8L:16D over 10 d. Temperature followed commercial recommendations, decreasing from around 30°C at bird level at 1-day-old to around 20°C by 4-wk of age. Chicks were given ad libitum water from bell drinkers for the full 24-h period and were fed chick starter 1 mash for the first 3 wk, chick starter 2 mash for the following 3 wk and then grower mash (all ABN, Cupar Mills, Fife; Female parent stock 4-stage rearing program, Aviagen, 2021) from 6-wk of age to the end of the trial. The feed was formulated in line with commercial broiler breeder standards. Food was provided ad libitum for the first 7 d and then in restricted amounts given at 08:00h each day that was gradually increased from 26 to 45 g per bird per day by the beginning of the 6th week, as per the Ross 308 parent stock guidelines (Aviagen, 2011). At 2 wk of age, all birds were weighed and wing tagged (10 mm × 10 mm padlock-style tags, Roxan Developments Ltd., UK).

At 6 wk of age, all birds were weighed and allocated to treatment pens (6 per diet) in groups of 4 birds according to an experimental design based on body weight (see below). Starting in the 6th wk of age, birds began to receive the experimental diets (see below). Lighting was also increased to 10L:14D hours light:dark at this point to allow enough light hours to complete all the necessary training and testing. All birds were weighed approximately weekly from 2 wk of age to the end of the trial (12 wk of age). Behavioral and physiological measures (see below) were collected during lights on (8:00−18:00).

Treatments

This experiment was designed to investigate possible effects of fiber on previously established behavioral and neuroendocrine measures (Dunn et al 2013; Dixon et al 2014). To do this we fed birds on 4 feed treatments from 6 wk of age: R birds were fed a standard broiler breed grower diet (basal diet) at the commercial recommended restricted feed ration quantity (Aviagen, 2011) which changed accordingly week by week; OH20% birds were fed the same basal diet amount as R birds but mixed evenly with a quantity of ground oat hulls (Hogarth Mills, Kelso) such that 20% of the feed by weight was oat hulls, OH40% was the same as the OH20% diet except that instead 40% of the feed by weight was oat hulls, and AL was unrestricted (ad libitum) access to basal diet. Oat hulls are known to be an indigestible filler, consisting of insoluble fiber, which chickens cannot digest (Hetland et al 2004); therefore the OH20% and OH40% treatments provided similar amounts of energy to the birds as the R diet if consumed at rates of 125% and 167% of R respectively. Practically, this meant that the birds on the basal diet received 45g/bird/d of feed at 6 wk of age and this gradually increased to 57g/bird/d at 12 wk of age; whereas the OH20% birds were given 56g/bird/d up to 71g/bird/d and the OH40% birds were given 75g/bird/d up to 95g/bird/d during this period. AL birds were fed the R diet but in ad libitum quantities.

Experimental Design

The 12 home pens in each of the 2 rooms were divided into 3 spatial blocks of 4 pens. Birds were systematically allocated to pens according to the rank of their 6 wk weight: to minimize within block variation birds were allocated to the 6 blocks ordered by their rank, and then to minimize between pen variation within blocks, birds were allocated 1 by 1 to the 4 pens successively by their rank. Finally the 4 feed treatments (R, OH20%, OH40% and AL) were allocated using 1.5 4 × 4 Latin Squares (with rows=block, and columns=pen weight order within block) which resulted in 6 pens and 24 birds in each feed treatment. Two observers recorded home pen behavior on 3 blocks each so observers were balanced with treatments, bird age and time in the day. For the foraging motivation behavior test, blocks of pens were allocated to 1 of 3 scheduling groups for which tests were staggered by 1 wk in such a way that each scheduling group contained 1/3 of the birds and the heaviest birds were tested first. These 3 scheduling groups and the use of 3 sets of apparatus were balanced with each other and with feed treatments. Bird post mortems were carried out block by block over 4 d when birds were age 85 to 88 d with heaviest blocks sampled first, as well as ensuring balance between the 2 teams who carried out the post mortems, treatments and sampling order.

Neurobiological and Physiological Measures

There were 2 teams each consisting of 3 people conducting the sampling between 13:00 and 17:00. 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 1.5 mL microfuge tubes (Sarstedt, Leicester, UK), 1 containing 100 μL 0.6M NaF/ 0.18M K Oxalate solution (for glucose measurements) and the other 50 μL Heparin (1,000 IU/mL) (for NEFA measurements). These tubes were mixed and then stored on ice for up to 1-h before being centrifuged at 8,000 g for 10 min at 4°C and the plasma removed and stored at -20°C until analysis. The bird was immediately euthanized with an overdose of IV pentobarbital. Once death had been confirmed, digestive organs which potentially would be affected by fiber inclusion; proventriculus, gizzard, pancreas, liver, and gall bladder and if applicable their contents were removed and weighed. The basal hypothalamus was dissected as described previously (Dunn et al., 2013). Presence of food, or not, in each of the crop, proventriculus, duodenum and ileo-caeco-colic junction (**ICCJ**) was recorded. Contents from the crop were weighed and scored on appearance: 1) Empty - no liquid or solid food evident, 2) Wet mush - mainly liquid with some soft solid food, 3) Solid mush - soft solid food, 4) Mix of dry pellets/solid mush - mainly soft solid food with few dry whole food pellets, 5) Dry pellets - whole dry food pellets, very little or no soft solid food.

RNA extraction and reverse transcription and measurement of anorectic (***POMC***) and orexigenic peptide (***AGRP***) gene expression in the basal hypothalamus were carried out by RTPCR as reported previously (Dunn et al., 2013).

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

Behavior- Home Pen Observations

All pens were video recorded for 24-h periods once a week for 4 wk either side of days when foraging motivation retraining and testing was taking place (see below). Each bird in a pen was individually identified by a pattern made with black livestock marker. Scan sampling was done by pausing the video every 5 min (total 12 scans) during five 1-h sessions throughout the light period and the behavior of each bird in a pen was recorded in each scan (from previous experience broiler breeders mainly rest/sleep during the dark period, Dixon et al 2022). This amounts to 23,040 behavior records, less 6.4% for which birds were not visible. The behaviors recorded were inactive (standing, sitting, sleeping), walking (including running, jumping, flying), foraging (pecking and scratching at litter), feeding (pecking at feed/feeder), drinking (pecking at and swallowing water), preening (while sitting or standing), dustbathing, object pecking (pecking at feeder, drinker, pen walls), aggressive pecking (peck directed to the head of another bird, delivered in a sharp, downwards manner), nonaggressive pecking (gentle and severe feather pecking, pecking at another bird's beak), and other (wing flap, shake, stretch, bill wipe) (Supplementary Table 1). We were unable to discern when all the feed was consumed from the feeder from the videos, therefore it is possible that birds were pecking at dust left in the feeder and not consuming feed when the behavior "feeding" was recorded. Anecdotal observations by the corresponding author who regularly fed the birds found that the majority of the feed was consumed in about 10 min for the R treatment, 15 min for the OH20% treatment and 30 min for the OH40% treatment. The AL treatment always had food in the feeder. Feeding began at 8:00 every day and took approximately 15 min to complete.

Behavior- Foraging Motivation Test

This foraging motivation test has been described in detail in Dixon et al (2014). At 6 wk of age, birds began habituation training to the foraging motivation apparatus (Figure 1). Habituation involved placing birds in the apparatus in small groups for 2 sessions each lasting 20 min, before being put in the apparatus alone for 2 further 20 min sessions over a period of 6 d, with each session being on a different day. Wood shavings were not present during this habituation phase but the full length of the apparatus as well as the start and (empty) wood-shavings platform and ramps were present.

There were 3 further training stages carried out over the next 6 d, each lasting 20 min, this time with wood shavings present, with each session on a different day. Birds did not have access to the whole length of the apparatus during training and were confined to the locations listed below. At each stage a bird had to reach the wood-shavings platform to move on to the next stage, otherwise that stage was repeated. This differed from the original protocol in Dixon et al (2014), which had 10 min training sessions, to allow birds more time to find the wood-shavings platform and hopefully reduce the number of birds that required additional training sessions. The stages were: 1) The start platform and wood-shavings platform were adjacent, without ramps in place. 2) Start platform and wood-shavings platform with ramps in place were adjacent. 3) As 2, but with

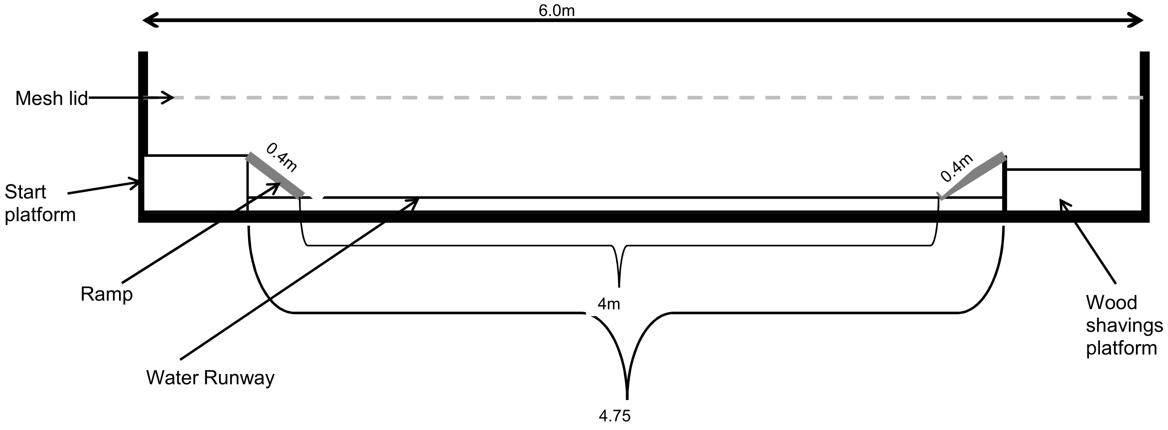

Figure 1. Diagram of the water runway apparatus. Birds were placed on the start platform and could chose to walk down the ramp into the water runway, up the second ramp and go onto the foraging area. The foraging area could be moved along the runway so that the distance travelled through the water could be increased up to a maximum of 4m. An adjustable mesh lid covered the apparatus to prevent birds from flying from the start platform to the foraging area.

20 mm deep water present in the water runway between the start platform and wood-shavings platform. In stages 2 and 3, the water runway was approximately 0.8m, although birds could choose to stay on the adjacent ramps and not enter the runway.

During testing (birds 9−11 wk of age), an increasing depth and distance (between ramps) of water was present in the runway. Birds could choose to remain on the start platform and ramp, or cross the water to access the platform containing wood shavings but no food where they could perform appetitive foraging behavior (pecking and scratching). Each bird completed 4 tests on consecutive days. Runway length was respectively 0.8 m, 1.6 m, 2.4 m, and 3.2 m; and depth was 1/3, 2/3, 3/3 or 4/3 of the birds' leg length (average over birds of each feed treatment to be tested in each week) for tests 1 to 4 respectively. This was done rather than using a standard depth, because the birds were growing at different rates, so a water depth "cost" relative to body size seemed more appropriate. Each bird's legs were measured from the ground to the top of the hock the day before the first test began each week.

Each test lasted 20 min. Testing was conducted between 10:00 and 17:00 based on the results of Dixon et al (2022) which investigated the effects of time of day and time since last meal on the motivation tests and found this time frame to have results where the effects of these factors would be minimized. At the beginning of a test, a bird was placed on the start platform and could spend the test time in whatever areas of the apparatus she chose to. After the 20 min 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 the test.

All birds participated in all tests, even if they had not been successful in getting to the wood shavings platform in their previous test. These tests were video recorded and test success (did the birds reach the wood shavings platform), latency to reach the wood shavings platform, time spent on the wood shavings and start platforms as well as durations of each visit and behavior (standing, sitting, preening, foraging, walking, other) on the start (tests 1 and 4 only) and wood shavings platforms were measured (Supplementary Table 1).

Ethical Considerations

Birds never had their water intake restricted and were housed on a bedding of wood shavings to provide comfort, insulation and allow for dustbathing behavior. The levels of food restriction we imposed were similar to or less severe than that used routinely in the poultry industry. However, ad libitum-fed broiler breeders can suffer from health problems and mortality (Renema and Robinson, 2004), therefore our birds did not begin the ad libitum feeding treatment until they were 6 wk of age and the experiment was ended when birds were 12 wk old, at which age they were still active and healthy. If any bird were to become lame and unable to reach food or water during the trial, they would be culled. However, all birds in the trial maintained good leg health for the duration of the study and this measure was not necessary. All procedures in this experiment were carried out under Home Office License and with the SRUC Animal Experiment Committee's approval. Birds were checked on at least 3 times per day.

Statistical Methods

Bird Growth. A linear mixed model (LMM) was fitted to the weekly live bird weights (log transformed) from ages 6 to 12 wk. Random effects included were room, block, individual pens of birds and the different sampling days within each pen, and individual birds and the different sampling days within each bird (the residual). A power law covariance structure was used to model errors being more highly correlated for individual birds for times closer together than for times further apart. Fixed effects were included for age (modelled as a classification with 11 levels), feed treatment (AL, OH40%, OH20%, R) and the interaction between age and feed treatment.

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

Random effects were included for block, pen, the 4 different days on which PMs were done within pens and individual birds (the residual variability). Fixed effects were included for the 2 PM teams (main effect only) and for age (modelled as a classification for 85, 86, 87, 88 d), feed treatment (AL, OH40%, OH20%, R) and the interaction between age and feed treatment.

For LMMs models were fitted to all data and also to data omitting outliers (as defined by visual inspection of the linear mixed model residual plots). This confirmed that results for all data are not just attributable to the outliers and so only analyses of all data are reported here. GLMMs with logit link function, binomially distributed errors and simplified fixed and random effects were also investigated for the binary variables (food in the crop, proventriculus, duodenum, ICCJ), but data was too sparse, so results are based on LMMs, which are a reasonable approximation in this case as the sample size is large. However, GLMMs that could be fitted confirmed results from LMMs.

Behavior - Home Pen Observations. Classifications from the original ethogram of behaviors statistically analyzed were inactive (standing, sitting, sleeping), walking (including running, jumping, flying), foraging (pecking and scratching at litter), feeding (pecking at feed/feeder), drinking (pecking at and swallowing water) and preening (while sitting or standing). Dustbathing, object pecking, aggressive pecking, non-aggressive pecking and other behaviors occurred too rarely to be statistically analyzed. For each of these classifications, the data was summarized over the 12 scans per session into tables of counts by the classes for each bird in each session, prior to subsequent statistical analyses. So that is 20 tables per bird (4 wk by 5 sessions per 24 h period). These tables of counts were constructed both including the not visible class and excluding it. Initial data exploration for the 6 resulting classifications suggested that exclusion of not visible birds had no impact on the results and so results presented here exclude these scans.

In order to analyze 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 totals for 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 block, 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, all fitted as categorical factors, were included for the observer (main effect only) and for the week of observation (a proxy for bird age), the time during lights on (8:30−9:30, 10:30−11:30, 12:30−13:30, 14:30−15:30, 16:30−17:30 h) and feed treatment (AL, OH40%, OH20%, R) and all interactions. The data was sparse for drinking and preening, most likely due to the use of scan samples and these being shorter behaviors in duration, so the 3 way interaction was omitted from the fixed effects.

Behavior - Foraging Motivation Test. Linear mixed models (**LMM**) were fitted to latency to reach the wood shavings platform (defined as the censored value, the total test time, for unsuccessful tests) and durations on the start platform and wood shavings platform, calculated as a proportion of total test time (all angular transformed). LMM were fitted to durations for different behaviors (standing, foraging, walking) exhibited on the wood shavings platform for successful birds and different behaviors (standing or sitting, standing, sitting, preening, foraging, walking) exhibited on the start platform for all birds at test numbers 1 and 4 only calculated as a proportion of time spent there (all angular transformed).

A generalized linear mixed model (**GLMM**) was fitted to the binary variable whether a bird successfully reached the wood shavings platform or not, with logit link function, binomially distributed errors and offset by total test time (log transformed). A GLMM was fitted to the counts of visits to the wood shavings platform by successful birds with log link function, Poisson distributed errors and offset by the time spent on the wood shavings platform (log transformed). Similarly, a GLMM was fitted to the counts of visits to the start platform by all birds with log link function, Poisson distributed errors and offset by the time spent on the start platform (log transformed). For these 2 GLMMs, the estimate of interest was the reciprocal of back-transformed means, that is, the average duration of platform visits.

Random effects were included for block, for individual pens of birds and individual birds, and, for LMMs only, the test numbers within pens. However, they were all fairly small apart from the variability between birds and between test numbers within birds (i.e., the residual variability for LMMs) for most response measures, as well as block for foraging on the wood shavings platform only, and pen for birds that successfully reached the wood shavings platform or not.

Fixed effects were included for the 3 apparatuses and the 2 testers (both main effects only) and the 4 test numbers, bird age (fitted as a 3 level factor), feed treatment (AL, OH40%, OH20%, R) and the time in the day at which birds were tested (fitted as a 4 level classification factor test start time ~10:00−11:10, 12:00−13:10, 14:00−15:10, 16:00−17:10) and all 2 way interactions apart from age by time in the day as this was confounded with tester. These models were fitted to 3 different subsets of the data (depending on availability of each response measurement): the whole data set, birds that successfully reached the wood shavings platform only or test numbers 1 and 4 only. Data was sparse for successful birds and so for analyses of birds that successfully reached the wood shavings platform or not, and analysis of measurements available only for successful birds, only main effects of test number and feed treatment were included.

Multi-Variate Analyses. Home pen behavior variables (proportions of behaviors) and key foraging motivation test variables (proportions) were summarized by calculating means over repeated measurements per bird to be included along with neurobiological and key physiological measurements in multivariate analyses of bird level variables. To explore bivariate relationships, Pearson's correlation coefficient (ρ) was calculated between these variables and scatter plot matrices of pertinent subsets of variables were shown by the feed treatments. Principle components analysis (**PCA**) was applied based on correlations giving loadings and biplots which show feed treatments, and the first 2 principal components were analyzed using LMMs with random and fixed effects as described in the above section on neurobiological and physiological measurements.

All Mixed Model Analyses. Random effects included in the LMMs and GLMMs described above are those that are biologically sensible, along with those that preliminary analyses suggested are non-negligible. Fixed effects were included that capture all remaining design aspects of the data, with effects of interest (feed treatment, time in the day for home pen behavior, test number for the foraging motivation test) and their interactions reported in the results. The other factors (e.g., apparatus, PM team, bird age, video observer), that are incidental to the main aims of the study, and some interactions with them, were included in the fixed effects to explain variation correctly, increase the power of the statistical analyses of the factors of interest and their interactions, and obtain unbiased means for effects of interest. The design ensured balance between most of these nuisance factors and the factors of interest. However, fixed effects were tested in sequence as listed above resulting in sequential statistical tests that adjusted for nuisance factors before testing effects of interest, ensuring robust conclusions about effects of interest.

P values are based on approximate F tests when available but otherwise are based on Wald tests. For F tests, F statistics are given in the results along with numerator and denominator degrees of freedom (ndf and ddf), whilst for Wald tests, Wald/ndf is given in order to make this comparable with F statistics, as the size of this statistic is an indicator of the strength of evidence of the effect. Model estimates of marginal means along with standard errors of means (±SEM), and standard errors of differences between means (**SEDs**) for effects of interest were obtained from the models which average appropriately across the levels of nuisance factors. Where modelling is on a transformed scale, means ± SEMs were back transformed onto the original scale to aid interpretation. Post hoc tests between estimated means for main effects 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. Post hoc tests for interactions of feed treatment with time (age for weekly live weights, time in the day for home pen behaviour, or test number for the foraging motivation test) were based on F tests for the interaction between time and the contrast between each pair of feed treatments, thus directly addressing whether there were differences in the trend over time between pairs of feed treatments.

One male bird (diet AL) was excluded from all statistical analyses. All data was compiled in MS Excel. Genstat 18 was used for the study design, data processing and all statistical analyses.

RESULTS

Bird Growth

Our efforts to ration birds to achieve similar weights in the 3 approximate to iso-energetic feed treatments (R, OH20% and OH40%) were successful. The highly significant interaction between age and feed treatment ($P < 0.001$, Figure 2) is largely due to increased growth in AL birds. Even though post hoc tests suggested significantly different trends with OH40% weights greater than R ($P = 0.008$), but not OH20% versus R or OH40% ($P > 0.05$), they remained at fairly similar weight throughout. At 6 wk of age, all 4 treatment groups were of similar weight ($P > 0.05$), but by 7 wk of age AL birds were already significantly heavier by about 131 to 134% on average than all other groups ($P < 0.001$) and by 12 wk of age they were about 215 to 221% heavier on average ($P < 0.001$).

Neurobiological and Physiological Measures

Organ Weights and Gut Contents. There were no significant interactions between bird age at post mortem and feed treatment for weight at PM, organ weights or gut contents ($P > 0.05$).

All organs (liver, pancreas, proventriculus, and gall bladder) with the exception of the gizzard were significantly heavier in the AL birds compared to the other 3 feed treatments as was bird weight at PM ($P < 0.001$, Table 1) and these are all positively correlated ($\rho \geq 0.66$). Gall bladders were also significantly heavier for OH20% than for R ($P = 0.026$). Gizzard weight was less correlated with the other organ weights and bird weight ($\rho \leq 0.42$) and significantly higher for AL and OH40% birds followed by OH20%, with R birds having the lightest gizzards ($P < 0.001$, Table 1).

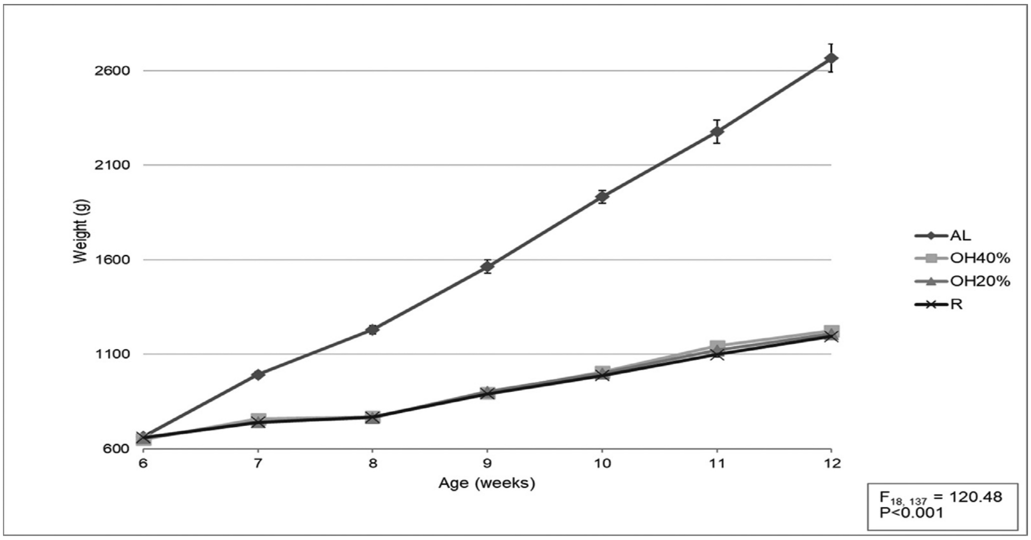

Figure 2. Bird weights for the 4 feed treatments from ages 6 to 12 wk. Values are back-transformed means ± SEMs estimated from LMM fitted to log transformed live bird weights. The F test is shown for the interaction between age and feed treatment.

There were no statistically significant differences in the weight of the crop contents or in the passage of food throughout the gastrointestinal tract (presence of food in the crop, proventriculus, duodenum, ICCJ) between the 4 feed treatments ($P > 0.05$, Table 1). The appearance of the feed in the crop was also similar between the 4 feed treatments, mainly appearing to be a solid mush or mix of dry pellets/solid mush at the time of culling ($P = 0.216$, Table 1).

Blood and Neuroendocrine Measures. Notably there was a highly significant effect ($P < 0.001$) of PM team on glucose measured in the blood plasma, and on both *AGRP* and *POMC* expression measured in the basal hypothalamus, but as PM team was balanced with all other fixed effects, this has no bearing on results apart from its inclusion as a main effect likely increases power of the tests of fixed effects of interest.

There were no significant interactions between bird age at post mortem and feed treatment ($P \geq 0.6$) for blood and neuroendocrine measures, apart from for NEFA ($P = 0.048$), but this is marginal so is not reported further.

Feed treatment had no statistically significant effect on NEFA or glucose measured in the blood plasma ($P \geq 0.138$, Table 1). *AGRP* mRNA was on average over 12 times higher in the hypothalamus of R, OH20% and OH40% birds than in the hypothalamus of AL birds ($P < 0.001$, Table 1, Figure 3A) but there were no statistically significant differences between R, OH20% and OH40% birds. In contrast, whilst *POMC* mRNA was

Table 1. Effects of the feed treatments on physiological measurements.

	Feed treatment					
Physiological measures	AL	OH40%	OH20%	R	F[1]	*P*
Weight at PM (g)	2812^{b}	1348^{a}	1309^{a}	1324^{a}	356.42	<0.001
Liver (g)	64.5^{b}	27.1^{a}	27.0^{a}	27.6^{a}	127.95	<0.001
Pancreas (g)	5.12^{b}	2.60^{a}	2.53^{a}	2.69^{a}	79.37	<0.001
Proventriculus (g)	9.72^{b}	5.25^{a}	5.12^{a}	5.01^{a}	82.43	<0.001
Gall bladder (empty) (g)	0.316^{c}	0.178^{ab}	0.185^{b}	0.158^{a}	38.99	<0.001
Gizzard (g)	49.7^{c}	51.2^{c}	42.3^{b}	37.3^{a}	37.12	<0.001
Crop content weight (g)	32.2	49.7	42.2	36	1.40	0.281
Food in Crop	0.958	1	1	1	1.04	0.378
Food in Proventriculus	0.36	0.083	0.169	0.21	1.77	0.224
Food in Duodenum	0.997	0.958	0.917	0.917	0.74	0.533
Food in ICCJ	0.958	0.958	1	0.958	0.33	0.802
Crop content Score (1-5)	2.874	3.184	3.191	2.993	1.03	0.417
Plasma NEFA	0.0183	0.0158	0.0172	0.0152	0.70	0.576
Plasma glucose	11.0	11.3	11.4	11.1	2.21	0.138
AGRP (bh)	0.0301^{a}	0.3684^{b}	0.3717^{b}	0.3657^{b}	16.16	<0.001
POMC (bh)	153.7	91.6	90.3	88.3	1.29	0.330

Values are means estimated from LMMs. Where data were analyzed on a transformed scale the back-transformed values are shown for biological meaning. Means, SEMs and SEDs on transformed scales can be found in Supplementary Table 2.

bh = measured from the basal hypothalamus

Superscripted letters indicate where differences lie, with means in ascending order a, b, c, ... Treatments sharing a letter do not differ significantly from each other.

[1] ndf = 3, ddf = 6.7−78.

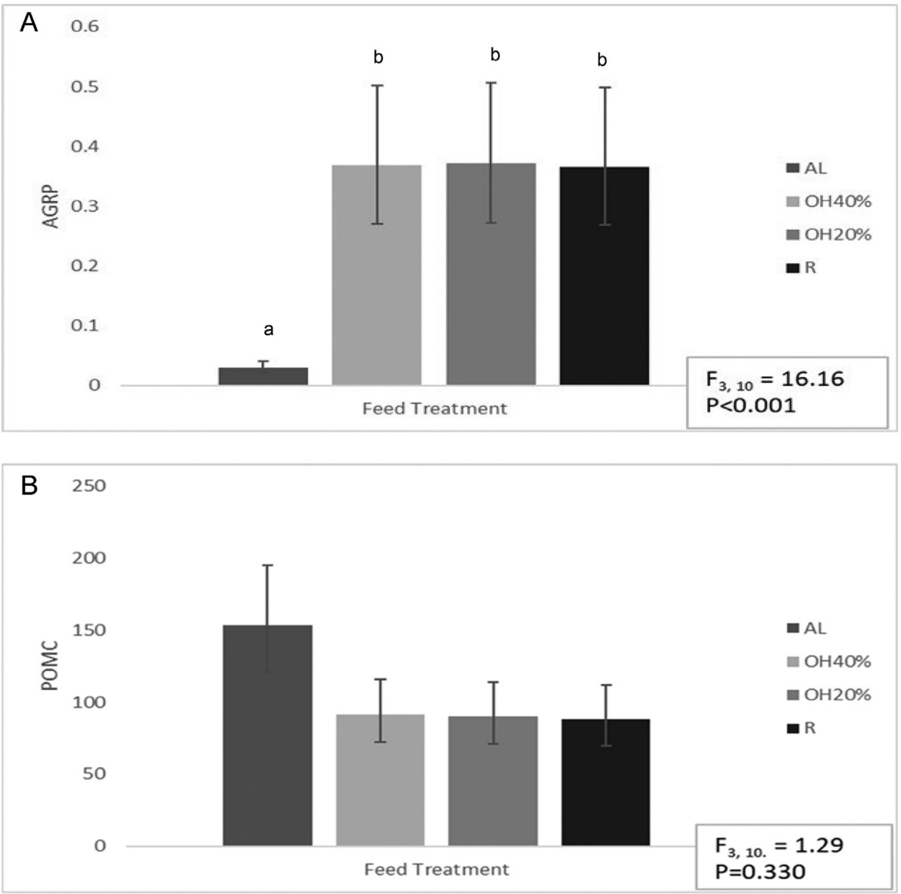

Figure 3. Effects of the feed treatments on AGRP (A) and POMC (B) gene expression in the basal hypothalamus. Values are back-transformed means ± SEMs estimated from LMMs fitted to log transformed gene expression measures. Letters indicate where feed treatment differences lie, with means in ascending order a, b, c, ... Treatments sharing a letter do not differ significantly from each other.

over 1.5 times higher in the hypothalamus of AL birds than in the hypothalamus of R, OH20% and OH40% birds, the diet effect was not statistically significant ($P = 0.330$, Table 1, Figure 3B). Correlations amongst blood and neuroendocrine measures are negligible, the largest being between glucose and *POMC* expression ($\rho = -0.30$).

Behavior- Home Pen Observations

There were some significant 3-way interactions and other significant effects of bird age for some behaviors, but effects are given here averaging over age effects, as this is not of primary interest. Averaging also over time in the day effects, AL birds spent a larger proportion of time inactive (standing, sitting, sleeping) ($P < 0.001$, Table 2 and Figure 4) than birds on the 3 restricted diets which did not significantly differ from each other ($P > 0.11$). Time spent feeding was affected by feed treatment ($P < 0.001$, Table 2 and Figure 4) and varied according to food volume (numerically AL>OH40%>OH20%>R), with AL birds spending significantly longer feeding than OH20% and R ($P = 0.016$, $P < 0.001$, respectively), and OH40% birds also spending significantly longer feeding than R ($P = 0.001$). There was a significant time of day by feed treatment interaction for feeding behavior ($P < 0.001$, Figure 5C). OH40% then OH20% then R all fed the most when new food was available in the morning, and this declined sharply by the middle of the day. In contrast, AL birds fed at a steady rate throughout the morning and feeding picked up at the end of the day prior to lights out. There was a significant time of day by feed treatment interaction for inactive behaviors ($P < 0.001$, Figure 5A), with inactivity for feed

Table 2. Effects of the feed treatments on home pen behavior averaged over time in the day and bird age.

	Feed treatment					
Home pen behavior	AL	OH40%	OH20%	R	F[1]	*P*
Inactive (Standing, Sitting, Sleeping)	0.331^{b}	0.104^{a}	0.136^{a}	0.140^{a}	16.51	<0.001
Walking	0.042^{a}	0.060^{b}	0.064^{b}	0.089^{c}	8.46	<0.001
Foraging	0.090^{a}	0.274^{b}	0.268^{b}	0.326^{b}	23.34	<0.001
Feeding	0.320^{c}	0.229^{bc}	0.182^{ab}	0.126^{a}	9.44	<0.001
Drinking	0.024	0.024	0.037	0.052	2.54	0.096
Preening	0.080^{c}	0.028^{a}	0.051^{b}	0.048^{b}	15.36	<0.001

Back transformed values (proportions) are shown estimated from GLMMs. Means, SEMs and SEDs on transformed scales estimated from GLMMs can be found in Supplementary Table 3.

Superscripted letters indicate where differences lie, with means in ascending order a, b, c, ... Treatments sharing a letter do not differ significantly from each other.

[1] ndf = 3, ddf = 15−147.

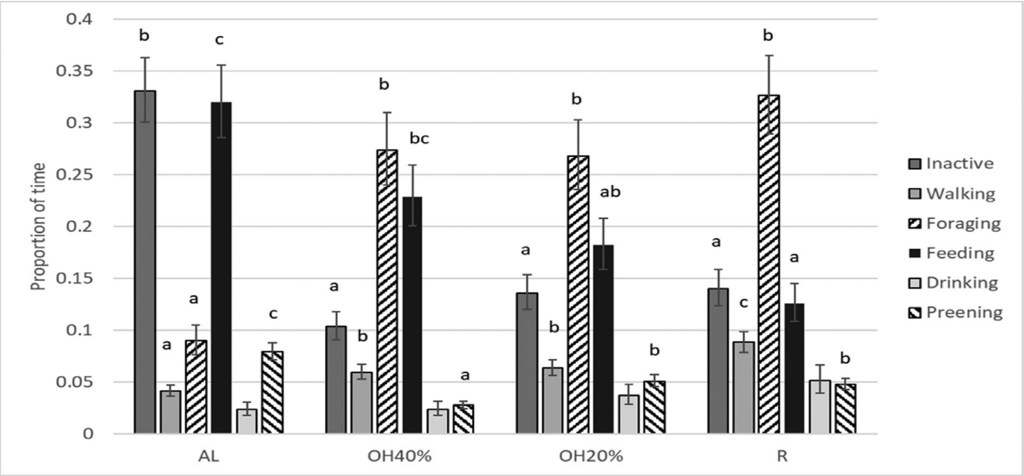

Figure 4. Proportions of time spent in the home pens Inactive (Standing, Sitting, Sleeping), Walking, Foraging, Feeding, Drinking and Preening for the 4 feed treatments. Proportions are back-transformed means±SEMs estimated from GLMMs with logit link. Letters indicate where feed treatment differences lie for each behavior, with means in ascending order a, b, c, ... Treatments sharing a letter do not differ significantly from each other.

restricted diets very low in the morning and increasing in the afternoon, particularly for birds feed R and OH20%. Averaging over time in the day effects, birds fed the restricted diets spent more time foraging than AL birds ($P < 0.001$, Table 2 and Figure 4) but did not differ significantly from each other ($P > 0.24$). There was also a

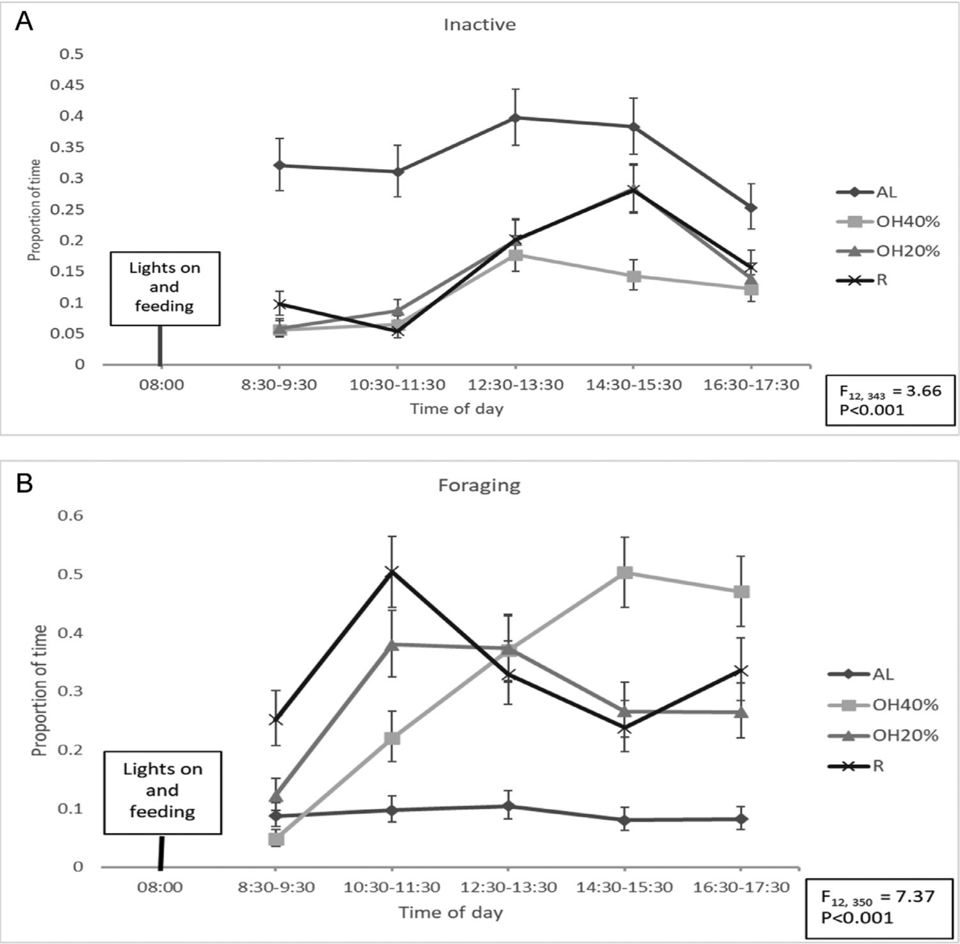

Figure 5. Proportions of time spent in the home pens (A). Inactive (Standing, Sitting, Sleeping), (B). Foraging, (C). Feeding and (D). Preening throughout the day for the 4 feed treatments. Proportions are back-transformed means±SEMs estimated from GLMMs with logit link. F tests are shown for the interaction between time of day and feed treatment. Post hoc tests of the trend with time in day between pairs of feed treatments indicate that for inactive (A) and preening (D) the time in the day effect is the same for R, OH20% and OH40 but differs for AL, whilst it differs between all feed treatments apart from AL and R for foraging (B) and R and OH20% for feeding (C).

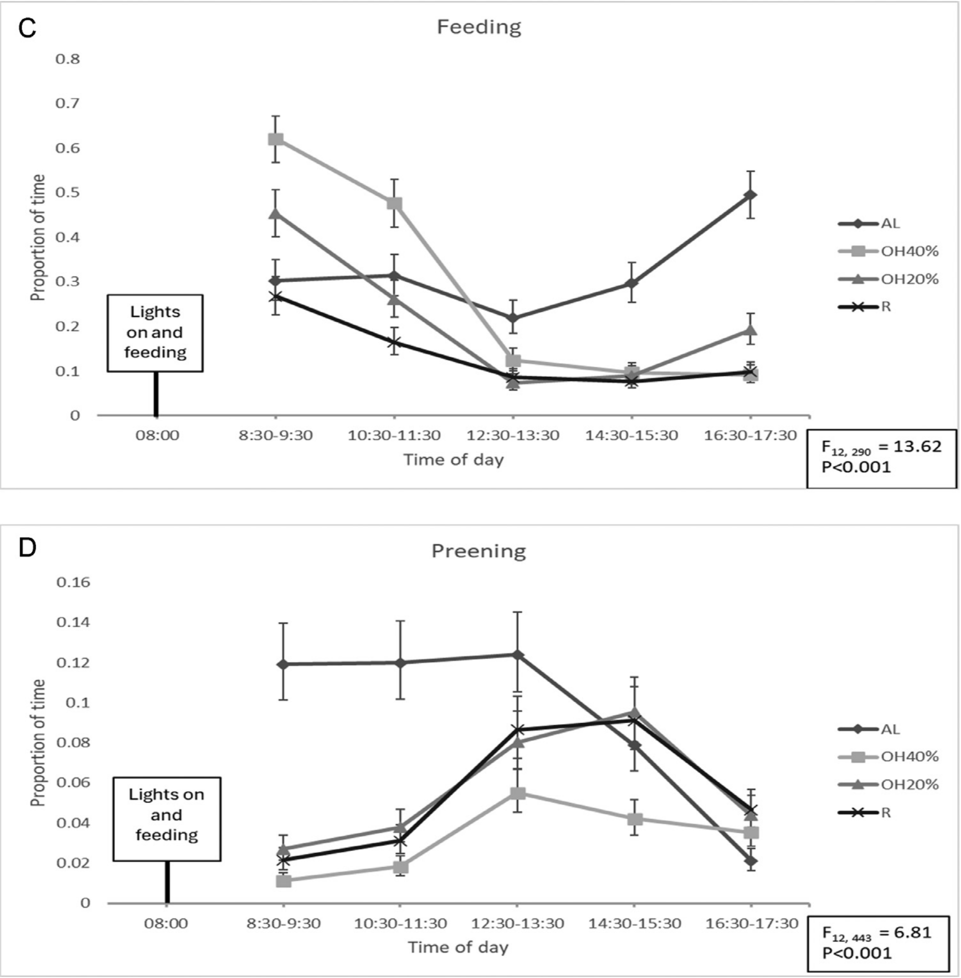

Figure 5 Continued.

significant time of day by feed treatment interaction for foraging behavior ($P < 0.001$, Figure 5B). R and OH20% showed peaks in foraging behavior mid-morning, followed by a decline and OH40% peaks in foraging behavior mid-afternoon and this remained steady until lights off, while AL birds showed a constant lower level of foraging.

Walking, preening and drinking were relatively infrequent but walking and preening were both significantly affected by feed treatment ($P < 0.001$, Table 2 and Figure 4). There was a trend for the amount of time spent drinking to increase with decreasing food volume, with R>OH20%>OH40%≈AL ($P = 0.096$, Table 2 and Figure 4). AL birds spend least time walking ($P \leq 0.005$), and R birds spent more time walking ($P \leq 0.046$), with walking intermediate for OH20% and OH40% birds ($P = 0.830$). AL birds preened significantly more frequently than those on the other 3 feed treatments ($P \leq 0.003$), whereas OH40% birds preened significantly less than R and OH20% ($P \leq 0.004$). There was a significant time of day by feed treatment interaction for preening behavior ($P < 0.001$, Figure 5D). AL birds decreased their preening in mid-afternoon while the other feed treatments showed increases in preening around this time, particularly R and OH20% birds. Similar results were found when preening was combined with dustbathing, which was too rare to analyze on its own. Object pecking was numerically higher in OH20% and R groups than in AL and OH40% but was too infrequent for a valid statistical analysis.

Behavior- Foraging Motivation Test

There was a marginally significant effect of apparatus on proportion of test spent on the start platform ($P = 0.025$) and latency to the wood shavings platform ($P = 0.029$), but as apparatus was balanced with all other fixed effects, this has no bearing on results apart from its inclusion as a main effect likely increases power of the tests of fixed effects of interest.

Whilst the percentage of successful tests overall showed the pattern expected with increased satiety due to gut fill (raw data summary statistics AL: 1.1%, OH40%: 7.3%, OH20%: 10.4%, R: 12.5%) willingness to cross was generally lower than anticipated in restricted diets. There was no significant effect of feed treatment (Table 3) on the proportion of birds that successfully reached the wood shavings platform ($P = 0.332$) or on the latency to reach this platform ($P = 0.148$). Whilst birds fed the AL diet spent a larger proportion of the test on the start platform (84%), than birds on the 3

Table 3. Effects of the feed treatments on the foraging motivation test measurements. Supplementary Table 4

	Feed treatment					
Foraging motivation test measurements	AL	OH40%	OH20%	R	F or Wald[1]	P
Foraging test success (proportion of birds) (GLMM)[2]	0.008	0.035	0.065	0.080	*3.42*	0.332
Latency to wood shavings platform (s)	1200	1195	1187	1175	2.18	0.148
Proportion of test spent on start platform	0.836	0.634	0.615	0.620	1.41	0.306
Proportion of test spent on wood shavings platform	0.000	0.001	0.002	0.003	1.41	0.246
Average duration of Start Platform visits (s) (GLMM)[3]	347^{b}	127^{a}	103^{a}	76^{a}	*16.23*	0.001
Successful birds: Average duration of Wood Shavings Platform visits (s) (GLMM)Ŧ§	409	82	43	44	*2.70*	0.440
Proportion of time on the start platform spent standing (test numbers 1 and 4)	0.450	0.689	0.651	0.611	2.94	0.081
Proportion of time on the start platform spent sitting (test numbers 1 and 4)	0.291^{b}	0.026^{a}	0.020^{a}	0.002^{a}	10.74	0.003
Proportion of time on the start platform spent preening (test numbers 1 and 4)	0.000^{a}	0.007^{b}	0.009^{b}	0.006^{b}	3.33	0.035
Proportion of time on the start platform spent foraging (test numbers 1 and 4)	0.080^{a}	0.137^{ab}	0.161^{ab}	0.249^{b}	4.28	0.008
Proportion of time on the start platform spent walking (test numbers 1 and 4)	0.006^{a}	0.015^{a}	0.024^{ab}	0.043^{b}	4.06	0.016
Successful birds: Proportion of time on the wood shavings platform spent standingŦ	0.992	0.372	0.402	0.320	1.05	0.459
Successful birds: Proportion of time on the wood shavings platform spent foragingŦ	0.022	0.392	0.196	0.199	0.24	0.868
Successful birds: Proportion of time on the wood shavings platform spent walkingŦ	0.009	0.074	0.289	0.143	0.75	0.552

Values are means and SEMs estimated from LMMs or GLMMs. If the data were analysed on transformed scales, back-transformed values are shown in brackets for biological meaning. Means, SEMs and SEDs on transformed scales estimated from LMMs or GLMMs can be found in Supplementary Table 4.

Superscripted letters indicate where differences lie, with means in ascending order a, b, c, Treatments sharing a letter do not differ significantly from each other.

Wald tests used.

[1] ndf = 3, ddf = 4−68.

[2] Only main fixed effects test number and feed treatment included.

[3] Analysed as Poisson count of visits, log link, offset log(duration).

restricted diets (~61 to 63%) this was not statistically significant (Table 3, $P = 0.306$). Feed treatment did not significantly influence the proportion of the test spent on the wood shavings platform which was low, on average, for all birds ($P = 0.246$). Whilst average duration of wood shavings platform visits by successful birds was lower for birds fed restricted diets than birds fed AL this was not statistically significant (Table 3, $P = 0.440$). Successful birds fed restricted diets on average spent less of their time on the wood shaving platform standing and more time foraging and/or walking than birds fed the AL diet but this was not statistically significant ($P \geq 0.459$). Sitting or preening on the wood shavings platform were each too rare for statistical analyses. Note that <8% of bird tests were successful, so power to detect effects based on this data subset is weak. Birds fed the AL diet had, on average, longer durations of start platform visits ($P = 0.001$) where they spent more time sitting ($P = 0.003$), and less time preening ($P = 0.035$) than the other feed treatments. Birds fed the R diet spent more time foraging (pecking and scratching at the wooden floor) on the start platform than AL birds ($P = 0.008$) with OH20% and OH40% birds not significantly differing in foraging behavior on the start platform from any of the feed treatments. R birds also spent more time walking on the start platform than AL or OH40% birds ($P = 0.016$) with OH20% birds not significantly differing in foraging behavior on the start platform from any of the feed treatments. In summary, whilst estimated means for many of the measurements from the foraging motivation test (Table 3) showed patterns expected with increased satiety due to gut fill (e.g. for foraging test success and for time spent foraging on the start platform R>OH20%>OH40%>AL) statistical evidence broadly only suggested AL birds were distinct from the other 3 diets.

When costs to reach the wood shavings platform increased (Supplementary Table 5), birds increased the proportion of time spent on the start platform and the average duration of visits ($P < 0.001$) after test 2 and decreased the time spent on the wood shavings platform ($P = 0.004$) after test 1. There were significant interactions between test number and feed treatment for the proportion of time spent on the start platform ($P = 0.046$) and the average duration of visits ($P < 0.001$). Birds increased the proportion of time spent on the start platform for all feed treatments apart from AL (Supplementary Figure 2A) and AL birds only decreased average duration on the start platform in test 3 (Supplementary Figure 2B). Birds also decreased their standing ($P = 0.007$) and increased their preening ($P = 0.009$) and foraging ($P < 0.001$) on the start platform from test 1 to test 4 (Supplementary Table 5) but behavior of successful birds on the wood shavings platform was not significantly affected by test cost, but, as mentioned above, power is weak. For the key measures, foraging test success and latency, the trend was as expected, with success decreasing ($P = 0.177$) and latency increasing broadly ($P = 0.223$) with increasing cost but these results were not statistically significant.

Multi-Variate Analyses

Pairwise Pearson's correlation coefficients are shown in Supplementary Table 7 for 20 key variables at the bird level used in the multivariate analyses (Supplementary Table 6). The largest correlations are seen between bird and organ weights, especially between the bird weights and liver, pancreas and proventriculus weights ($\rho \geq 0.88$), and then with empty gall bladder weights (ρ

≥ 0.67), and then gizzard weight ($\rho \geq 0.30$). A scatterplot matrix (Supplementary Figure 3) suggest that the stronger associations are apparent between birds within feed treatments as well as between feed treatments for which any difference is due to AL. The direction of the effect of the feed treatments on crop content and gizzard weight are similar (R< OH20% < OH40%) apart from for AL, and the resulting correlation is small ($\rho = 0.19$) with no apparent associations between birds within feed treatments. AGRP is negatively associated with bird and organ weights ($\rho = -\ 0.76$ to -0.60, $\rho = -0.31$ for gizzard) marginally apparent between birds within feed treatments as well as between feed treatments for which any difference is due to AL (Supplementary Figure 3). POMC is marginally positively associated ($\rho = 0.16$ to 0.25, except for with gizzard). Marginally negative associations of glucose with bird and organ weights appear merely due to decreased glucose on average in AL birds. Heavier birds are less active ($\rho = 0.66$), foraging, walking and drinking less ($\rho = -0.63, -0.43, -0.28$, respectively) and preening and feeding more ($\rho = 0.52$, 0.30, respectively) (Supplementary Table 7) and these negative associations appear largely due to differences in home pen behavior of AL birds (Supplementary Figure 4).

PCA of the 20 key variables (Supplementary Table 6) resulted in over 45% of the variation being explained by the first two principal components (Supplementary Table 8). The first principal component (**PC1**) explained 34.5% of the variation and the second principal component (PC2) 11.1%. A scatterplot matrix of the first 4 principal component scores (Supplementary Figure 5) suggests PC1 is capturing differences due to the AL diet whilst PC2 and PC3 appear to be distinguishing between R, OH20% and OH40%. This is confirmed by LMMs of PC1 and PC2 scores which both give significant effects of feed treatment (PC1: $F_{3,8} = 232.88$, $P < 0.001$, AL>OH20%≈OH40%>R; PC2: $F_{3,78} = 8.93$, $P < 0.001$, OH40%>AL≈OH20%>R). Loadings (Supplementary Table 9) and biplots for PC1 and PC2 (Supplementary Figures 6 and 7) shows that PC1 has large positive loadings for bird and most organ weights, inactive and preening (and smaller positive loadings for gizzard weight, feeding and duration of foraging tests spent on the start platform) and large negative loadings for AGRP and foraging (and smaller negative loadings for walking and feeding). By contrast PC2 has large positive loadings for latency to reach the wood shavings platform in foraging tests, crop content and gizzard weights, foraging, and glucose (and smaller positive loadings for feeding and NEFA) and large negative loadings for duration of foraging tests spent on the wood shavings platform, walking, preening and inactive. Thus, the indication is that the variables listed for PC2 are contributing to differences between the three restricted diets, especially crop content and gizzard weights and behavior in the foraging motivation tests.

DISCUSSION

As expected, birds fed ad libitum on a high quality commercial ration, grew faster and were significantly heavier than all of the other treatments, weighing over twice as much as the feed restricted birds by 12 wk of age. Adding fiber to the diets resulted in birds that grew broadly at the same rate (weighed the same) as birds fed a commercially restricted feed ration even though the daily feed portions were larger in the 20% and 40% oat hulls feed treatments. Almost all internal organ weights also reflected the body weight difference between the ad libitum and the 3 restricted treatments. Gizzard weight was the only exception - it was larger in AL (and OH40%) birds, but also showed differences within the restricted treatments, reflecting food volume/fiber content effects. Therefore, from a health perspective, the birds on the fiber treatments should still benefit physically from the slower growth rate and have lower incidences of lameness, metabolic issues and mortality than are found in broiler breeders fed larger quantities of feed (Mench, 2002).

Some aspects to the design of the current study, which were included as fixed effects in the statistical models (for example the team carrying out the post mortems, and the apparatus used in the foraging motivation test) were statistically significant on some of the associated measurements. The same was found in a previous similar study (Dixon et al., 2022). This reinforces the point that such aspects should be considered when designing these types of studies, to ensure there is no confounding of these factors with effects of interest, such as treatments, age and time in the day. Furthermore, their inclusion as nuisance factors in the statistical analyses, could increase the power to investigate effects of interest.

AGRP expression in the basal hypothalamus (which stimulates feeding behavior) was lower in AL birds compared to the 3 restricted treatments that did not differ. The relative difference in *AGRP* expression between AL and R was less in this study (around 12-fold using the back transformed values) compared to our previous work in which this difference has been over 60-fold (Dunn et al., 2013) and 20-fold (Dixon et al., 2022) but still represents a large difference. Therefore, it is clear that increased foraging behavior and increased *AGRP* expression in the hypothalamus occurs together. However, in some previous experiments, *AGRP* expression seems to be associated with the difference between a bird's actual vs potential body weight, whether that difference is imposed or is voluntary, for example as a result of broody behavior (Dunn et al., 2015), therefore high *AGRP* expression does not always result in increased food seeking behavior. Although in the absence of physiological states that result in voluntary anorexia such as broodiness, the relationship between food seeking behaviors and *AGRP* expression may be valid. The lack of a feed treatment difference in the 3 restricted diets here suggests that any feedback from the increased gut fill which results from the consumption of oat hulls makes little difference to *AGRP* expression. Although energy intake was not calculated in this study, it has been shown that increased *AGRP* expression primarily reflects the total energy intake in the short term (Caughey et al., 2018), or in the longer term the discrepancy between energy intake and the bird's internal growth target (Dunn et al., 2013; Dunn et al., 2015). One result of this is likely to be food seeking behavior (Aponte et al., 2011) which is shown as foraging in poultry as discussed above. Indeed, in mice the activation of AGRP neurons was completely sufficient to explain feed seeking behavior (Aponte et al., 2011), which relates to foraging motivation. *POMC* expression in the basal hypothalamus and plasma glucose did not vary significantly between feed treatments which is similar to what we have previously found for R vs AL diets in some studies (Dunn et al., 2013). There were also no treatment differences found in the current study for plasma NEFA. However, Dixon et al., (2022) found that the plasma NEFA differences between AL and R treatments were only large shortly before the birds' daily feed, with NEFA highest in restricted birds at the time furthest from feeding and as we avoided this time frame in the current study, this is likely to explain the discrepancy.

Feed restriction is known to be associated with increased activity (reduced inactivity) and foraging or litter-directed behavior (reviewed by Kostal et al., 1992; Savory and Maros, 1993; D'Eath et al., 2009). In the home pens, AL birds showed less walking, foraging and overall activity and more preening than R, OH20% and OH40% birds. Unexpectedly OH40% birds showed the least amount of preening with R and OH20% being intermediate between OH40% and AL levels. Birds that have behavioral needs (like hunger) fulfilled should have more time for comfort behaviors like preening (Dawkins, 2003). Although OH40% birds had larger daily rations of feed and spent more time feeding than R birds, they also had similar levels of foraging and overall activity to OH20% and R birds per day, meaning this may have left less time available to preen. Overall, the amount of preening observed in the home pens was low; however, this could be impacted by our sampling method. Preening can be a relatively short duration behavior (e.g., Li et al., 2020), compared to other behaviors, such as sitting. Behavior was recorded using scan sampling, which is a "snapshot" of behavior at a particular time (Bateson and Martin, 2021), therefore it is possible short preening bouts were harder to capture using our methods.

There were also time of day × treatment interactions for some behaviors: birds on the restricted diets fed the most when new food was available in the morning with a sharp decline in the middle of the day which probably reflects when the food runs out while AL birds fed at a more steady rate during the day with a slight increase before lights out. The increase in AL birds feeding before lights out is likely to be anticipatory feeding driven by photoperiod (May and Lott, 1992). The increase in feeding in OH20% birds in the last observation may represent misclassification of pecking at the empty feeder as feeding behavior. The interaction of time of day × treatment for foraging behavior may also relate to the timing of food running out in each treatment group. R and OH20% show peaks in foraging behavior mid-morning, followed by a decline which could reflect the point at which the birds "give up" on litter foraging as an actual (spilt feed) or potential food source. OH40% peaks in foraging behavior mid-afternoon and this remains steady until lights off at which point the birds become less active and mainly rest/sleep, whereas AL birds show a constant lower level of foraging, perhaps reflecting a basal level of "behavioral need," while most of their pecking was directed at food. Caloric restriction has been shown to affect timing and duration of behavior patterns in other species, such as mice (Acosta-Rodriguez et al., 2017). Mice fed 70% of their ad libitum intake consumed their daily allowance within an hour of presentation whereas mice undergoing temporal feed restriction (given ad libitum access to food for a 12 h per day) consumed 50% of their feed intake within 4 to 5 h after presentation (Acosta-Rodriguez et al., 2017). This indicates that the timing and size of the meal provided to broiler breeders could also impact the behavior performed throughout the day. The mice in Acosta-Rodriguez et al. (2017) received much larger proportions of their daily feed requirements compared to ad libitum animals than commercially reared broiler breeders and they were still motivated to consume all their feed quickly. This implies that even small levels of feed restriction may result in hunger and changes in temporal behavior patterns compared to fully fed individuals.

In the foraging motivation test, birds were less likely to cross the water to reach the wood shavings platform than they were in previous experiments (Dixon et al., 2014; Dixon et al., 2022). The AL treatment's success in reaching the wood shavings platform has also been low in our other studies (Dixon et al., 2022) and is an expected result since these birds should have low motivation to access the area. However, the R, OH20% and OH40% treatment birds also had a low success rate (ranging from 7.3 to 12.5%) compared to another trial using similar methods: birds fed the R diet at 1 of 2 times of day were successful in 52 to 56% of the tests (Dixon et al., 2022). The main difference between this study and our previous work was the birds in this study were provided feed in a mash form (instead of pellets) because the levels of fiber that were included would not pellet well. Mash takes longer to eat than pelleted feed and the increased feeding times may satisfy some foraging motivation (Nielsen et al., 2011). Also, mash would be easier for the birds to spill from the feeders which could make foraging on the ground in the pen more rewarding, and again, decrease motivation to forage elsewhere, especially when foraging in the apparatus was never successful. A direct comparison of birds given the same feed but in mash or pelleted form would help clarify this. Additional differences in methodology to Dixon et al. (2022) were in the current study training sessions lasted 20 min as opposed to 10 min to allow the birds more time to reach the wood shavings platform during training and reduce the number of birds that needed additional training sessions. However, perhaps the longer training periods allowed the birds to learn that food was never present on the wood shavings platform and decreased their motivation to access it (Bouton, 2004). The birds in the current study were tested when they were 9 to 11 wk of age while previously, they were tested at 8 to 11 wk of age (Dixon et al., 2022). Feed restriction increases to its most severe level around 11 to 12 wk of age (e.g., Aviagen, 2021) so the birds in the current study could have been on a similar feed restriction schedule and been similarly hungry (for the R treatment at least) as those in our previous study. Thus, we think that the difference in feed form is the most likely cause of the low response rate.

The AL birds had longer average visit durations to the start platform than the other treatments, which suggests that they mainly stayed on the start platform and did not make many trips to other areas of the apparatus, while the other feed treatments had shorter average visits to the start platform so made more frequent moves away from this area. Restricted fed birds are more active than ad libitum fed birds (e.g., Mench, 2002; de Jong and Guemene, 2011; Dixon et al., 2014) so this indicates that adding fiber to the diets did not significantly decrease the birds' activity in a relatively novel environment.

The success rate in the foraging motivation test was low, but we found differences in the birds' behavior on the start platform during the tests. AL birds spent more time sitting and less time preening than the R, OH20% and OH40% birds. Preening is usually classified as a comfort behavior that gets performed when other behavioral needs are met (de Jong et al., 2003; Nicol, 2015). In the home pens, as found in the current study although at low overall levels, preening is usually performed more by birds fed ad libitum compared to restricted diets (e.g., Dixon et al., 2022). Based on this, we would suggest the preening behavior carried out by restricted birds on the start platform is more likely displacement behavior, where a different behavior is performed than the one the animal is motivated to perform but cannot (Duncan and Wood-Gush, 1972; Kostal et al., 1992). The water runway apparatus is a relatively novel environment to the birds and one in which the birds must overcome aversive stimuli (walking through water) to reach the rewarding area (wood shavings platform). This may have resulted in birds who wanted to explore the wood shavings area but did not want to cross the water and as a result began displacement preening. The amount of preening on the start platform here was significantly higher for the higher cost test, consistent with the interpretation that the preening shown by the restricted feed treatments was a displacement activity. However, the actual change in proportion of preening behavior was small and may not be as biologically important. As opposed to the scan sampling used in the home pen data, all behavior performed by the test bird was recorded for frequency and duration on the wood shaving platform and for tests 1 and 4 on the start platform. Therefore, all instances of preening were recorded, making this a more accurate representation of the overall amount of preening done by birds in the test apparatuses. R birds spent more time foraging on the start platform than AL birds with the fiber treatments intermediate but not statistically significantly different from either. R birds also spent more time walking than the AL and OH40% treatments with OH20% intermediate but not statistically significantly different from the other feed treatments. A decrease in foraging and walking could indicate some positive behavioral effects of increased fiber diets (e.g., Arrazola et al., 2020).

For the principal components analysis, Principal Component 1 is what one might expect, with the largest effects related to diet in broiler breeders and is presumably being driven by the difference in body weight, with positive loadings for bird weight and most of the major organ weights. In addition, the anorectic hypothalamic neuronal AGRP gene expression has a large negative loading. We know that AGRP expression in the brain positively correlates with the distance a bird is from its target body weight (Dunn et al., 2013). Although it can also reflect short term experience of feeding (Dunn et al., 2013). The behavioral loadings are also what one might expect. There are negative loadings for foraging and walking, which increase with feed restriction and positive loadings for inactivity and preening, behaviors associated with satiety (D'Eath et al., 2009).

Principal Component 2 has some interesting characteristics. It appears to differentiate the three restricted diets with different fiber levels. It contains the upper digestive tract traits of crop content and gizzard weight. Gizzard weight differs from the other organ weight traits in that it responds to diet bulk (Hetland et al., 2003), perhaps in this case correlated with the crop content difference which is likely affected by the bulk of the diets. The metabolic factors, glucose and NEFA also have positive loading. Paradoxically the behavioral traits of foraging and feeding also have positive loading in PC2 despite the crop content and there are negative loadings for preening and walking. The foraging and feeding paradox could be due to the bulk of the low-density diet being associated with searching for more nutritious food, which was observed before with high fiber diets (Nielsen et al., 2011). As might be expected, latency to reach the wood shavings platform has a positive loading and may relate to differences between fiber content of the diets. The duration of time on the wood shavings platform has a negative loading, suggesting shorter visits and perhaps reflecting different activity levels for different fiber levels of the restricted diets (Nielsen et al., 2011). So, although the principal component analysis suggests some potential role of dietary dilution in changing behavior based on metabolic changes, this would certainly need more research to unravel and understand any relationship.

When taking all of the results found here into account, there is not a strong case for the addition of fiber improving satiety and overall welfare in broiler breeders. The behavioral and physiological measures therefore do not seem to indicate improvement of the subjective mental experiences (as an important component of welfare, Dawkins, 2008) of the birds. None of the measures alone provide good evidence for the indirect inference of the emotional states of the broiler breeders as many factors could affect their expression (e.g., Dixon et al., 2022); however, taken as a whole, they could allow insight into their subjective experiences. Higher fiber diets may improve litter quality and decrease severity of pododermatitis and hock burn though (Kheravii et al., 2018) which would improve other aspects of broiler breeder welfare aside from hunger. This is similar to the results of other studies which found no improvement in the fiber diets or only small changes in behavior when birds were provided higher fiber feeds (e.g. Zuidhof et al., 1995; Savory and Larivere, 2000; de Jong et al., 2005; Sandilands et al., 2005; Hocking, 2006; Riber and Tahamtani, 2020a; Tahamtani et al., 2020). Although a small change in feeding motivation when fiber is provided has been found in some studies (Riber and Tahamtani, 2020b) but not others (Riber and Tahamtani 2020a). Additionally, the amounts of fiber (oat hulls) added to the diets are not practical in a commercial system, especially the OH40% treatment because they are difficult to pellet. Therefore, exploring other dietary manipulations may be a better way forward. Diets lower than the commercial standard in protein and energy allow the birds to be given larger daily rations and have been found to reduce standing, walking, foraging and stereotypic object pecking (van Emous et al., 2014).

Another option would be to relax the feed restriction levels, allowing the birds to eat more and grow faster while still maintaining health and reproductive parameters. Broiler breeders reared up to a higher weight of 2,400 g at 20 wk of age (compared to the industry recommended 2200g) showed more sitting and less foraging behavior, and lower plasma cortisol at 16 wk of age compared to standard reared birds (van Emous et al., 2015). However, another study found that birds grown to a 15% higher growth curve did not show any difference in behavior to birds reared to the standard commercial growth curve aside from spending longer at the feeder (de los Mozos et al., 2017). An alternative to only relaxing restriction levels would be to genetically select for birds that can consume more food but continue to meet target growth rates. A similar strategy is being used in the breeding of slower growing broilers where slower growing or dwarf females are used as the parent stock. These birds can consume more food than a faster growing breeder female and maintain good health and reproductive abilities (e.g., Triyuwanta et al., 1998). Genetic variation does exist in feed consumption and growth rates within flocks of broiler breeders (Lindholm et al., 2017); therefore, selecting for parent stock that can eat more, be healthy and reproduce is possible. However, in practice, this could mean a number of changes to the current breeding programs. Additionally, the link between faster growing breeders who can consume more food while maintaining slower growth and production measures that need to be selected for to maintain productivity in the offspring are not clear.

In conclusion, the expression of *AGRP* in the orexigenic neurones of the hypothalamus increased in feed treatments which also increased foraging behavior, which in this scenario is likely to be linked to increasing food seeking behaviors. In terms of behavior, whilst estimated means for the 4 feed treatments exhibited the pattern expected with increased satiety due to gut fill for several measurements, there is not much evidence that adding fiber to broiler breeder feed rations which allow for larger daily portions statistically significantly improves the satiety of the birds. There were some small behavioral differences in the fiber diets (such as decreased walking) that could be considered positive effects but these differences were not enough to indicate a biologically significant improvement in bird welfare. Therefore, other methods of increasing satiety and improving bird welfare should be examined.

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DISCLOSURES

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SUPPLEMENTARY MATERIALS

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