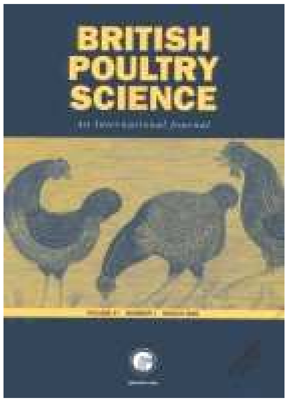

The use of conditioned place preference to determine broiler preferences for quantitative or qualitative dietary restriction

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TITLE PAGE

The use of conditioned place preference to determine broiler preferences for quantitative or qualitative dietary restriction

Running title: Conditioned place preference and hunger broilers

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1. Abstract

1. Calcium propionate (CAP) may improve the welfare of feed restricted broiler breeders by improving their satiety when included within the feed ration. However, the evidence for this is mixed.

2. This study used a closed economy conditioned place preference (CPP) task and aimed to identify whether broilers (as a model for broiler breeders) preferred an environment associated with quantitative food restriction (QFR) or an environment associated with a diet quality-adjusted by the inclusion ofCAP. Birds taught to associate different environments with QFR and*ad libitum* (AL)access to feed were used to validate the methodology.

3. The two treatment groups were 1) QFR/AL (n = 12) in which birds alternated every two days between QFR and *ad libitum* access to food, and 2) QFR/CAP (n = 12) in which birds alternated every two days between QFR and QFR + calcium propionate (increased from 3 – 9% over the study period). Birds were taught to associate one diet option with vertical stripes and the other with horizontal black and white stripes. Each bird was tested twice for a CPP (once per diet).

4. QFR/AL birds showeda significant preference for the pen associated with *ad libitum* access to feed, but onlywhen tested hungry (i.e. fed QFR on day of testing).QFR/CAP birds did not show a preference under either hunger state.

5. Reasons for the failure of QFR/CAP birdsto show a preference are unclear but could include a lack of preference or failure to learn the task.

6. The existence of state-dependent effects indicates that care is needed in the design of future CPP studies and that the effect of calcium propionate and level of hungeron ability to learn a CPP needs further investigation.

2. Introduction

Broiler breeders of fast growing strains of broilers (the most common commercial lines) are feed restricted to ensure that the bird has a healthy rate of growth and maximal rates of fertility (Savory*et* *al* 1993). This feed restriction is both severe, with birds fed as little as 25 – 45% of *ad libitum* intake (dependent on whether comparisons are made between age or bodyweight-matched birds; Savory*et al* 1993), and chronic, with birds being feed restricted to various degrees from about 1 week of age until the end of their productive life (circa 18 months; based on manufacturer performance objectives, Aviagen, 2007). The available behavioural and physiological evidence indicates that these birds experience chronic hunger (Savory*et al* 1993; Savory and Maros, 1993; Savory and Mann, 1999; Hocking *et al* 1993, 1996, 2004; De Jong *et al* 2003). With around 6.3 million broiler breeders being reared in 2010 alone in the UK (DEFRA, 2011) feed restriction is a major welfare issue within the meat bird industry.

Quality adjusted diets that take longer to consume potentially improve feed restricted broiler breeder welfare by increasing satiety and allowing more naturalistic foraging behaviour to occur (for a review see D'Eath*et al* 2009). However, there is a need for further research to quantify this perceived benefit by using methods that identify the relative affective state of the broiler when fed either a quantitatively or qualitatively restricted diet. One potential dietary adjustment that might improve levels of satiety is the addition ofpropionate-containing compounds such as calcium propionate (CAP). Propionate has been linked to increased feelings of satiety in humans Experimentally, the addition of CAPto broiler feed has been shown to reduce feed intake by up to 25% when fed to immature (4 – 8 week old) broiler breeders at a 3% inclusion rate (Kapkowska*et al* 2005) although this declines to about an 8% reduction in feed intake by 18 weeks of age. Sandilands *et al* (2006) achieved a larger voluntary reduction in feed intake by increasing the inclusion rate from 5 – 10% over the rearing period, although the bodyweight of these birds was still significantly greater than birds reared using quantitative feed restriction to commercial levelsat 6 and 12, but not 18 weeks of age.

However, the mechanism by which propionate achieves this reduction of food intake is unclear. It is thought to act by delaying gastric emptying and / or by various post-absorption effects on metabolism (Arora, *et al*2011; but see Darzi, *et al* 2011). One plausible hypothesis is that this results in a sensation of satiety (a positive affective state) which birds find rewarding. Alternatively though, birds may find eating or utilising food containing CAP unpleasant in some way. Darzi, *et al* (2008)found that when propionate was administered orally in a palatable form to humans there was no suppression of appetite. Metabolic acidosis might be induced at high inclusion rates, but at the low levels typically used this is not a problem (Pinchasov and Elmaliah, 1994). Oral lesions have been observed in some studies (Tolkamp *et al* 2005; Bolton and Dewar, 1964) suggesting oral discomfort as a mechanism but this is not always observed (Buckley *et al*, unpublished data). The fineness of mash diets, which is exacerbated by the inclusion of CAP,have also been implicated in the aetiology of oral lesions (Gentle, 1986; Tolkamp*et al* 2005). Studies which bypass the gastrointestinal tract by injection of propionic acid (the active ingredient) also achieve appetite suppression (Pinchasov and Elmaliah, 1989). Previous work by Buckley *et al* (2011) found that diets containing 3% calcium propionate were less preferred compared with an otherwise identical basal diet by broilers in a two-pan simultaneous choice test. However, this preference may be sensory-led and not reflective of the affective state of the bird post ingestion (i.e. does it increase satiety relative to quantitative dietary restriction?). To summarise, the effect of CAP on feed intake might be a consequence of increased satiety (a positive affective state) or alternatively result from an aversion that results in the animal delaying consumption despite being hungry (a negative affective state). This issueneeds to be addressed before a claim that CAP improves the welfare of feed restricted broiler breeders can be made.

The current study uses Conditioned Place Preference (CPP) to investigate whether broiler chickens find food containing CAP aversive or rewarding. CPP methodologies are based on the principles of Pavlovian conditioning: an animal can be conditioned to prefer a previous neutral, or un-preferred, environment by pairing it with the presence of something that the animal finds rewarding (Tzschentke, 1998). It is an approach widely used within the pharmaceutical industry (reviewed by Tzschentke, 1998; Bardo and Bevins, 2000)to investigate the effects of various pharmaceutical agents on the affective state of the animal. The animal is injected with the compound and then immediately placed within the distinctive environment to be conditioned. If the drug results in a positive affective state then the animal will prefer this environment over one it is placed into after an injection of saline (which has no effect on affective state). However, CPP has also been demonstrated to occur as a consequence of natural reinforcements including food (e.g. Spyraki*et al* 1982; Papp, 1988; Papp *et al* 1988, 1989; Imaizuma, *et al* 2000, 2001; Dickson *et al* 2010; Matsumura *et al* 2010). Whilst most studies are in rodents, CPP have been demonstrated in avian species including quail (Mace *et al* 1997;

Akins et al 2004) and chickens (Bronson *et al* 1996; Hughes *et al* 1997). Recent work by Dixon *et al* (2011) suggested feed restricted broiler breeders can learn a CPP using aversive stimuli that they are exposed to for several days in a closed economy environment. Y- or T-maze type choice tests in which feed restricted broiler breeders have to choose between different quantities or qualities of food that were associated with distinctive arms of the maze proved unsuccessful in previous research (Buckley *et al* 2011a, 2011b). Thus, it is appropriate to investigate methods in which the animal is in contact with the to-be-conditioned stimuli for longer (e.g. at least eight days, Dixon *et al* 2011)compared to the time spent in a Y-maze arm (which may be as little as 2 seconds in commercial feed restricted broilers before they make a choice and exit the maze, Buckley *et al* 2010b). Also potentially beneficial is that in CPP testing the animal is tested in extinction (i.e. with no food present that is likely to elicit impulsive behaviour).These methods may be more successful at determining broiler breeder preferences for quantitative or qualitative dietary restriction. Further, training and testing the animal in a closed economy conditioned place preference apparatus may allow preferences based on the whole experience of each diet 'system' to be identified. This isbecause the animal will be in contact with the to-be-conditioned stimulus post-consumption of the diet. Thus, the animal should express a preference based on its overall affective state (primarily how hungry or satiated the bird felt post-diet consumption) on days during which it experiences quantitative (or qualitative) dietary restriction.

The current study hypothesised that feed-restricted broilers would show a preference when given a choice between environments associated with quantitative dietary restriction (QFR) or qualitative dietary restriction (diet containing CAP) in a closed economy CPP task. It was expected that the direction of this effect would indicate whether the broilers found a diet qualitatively-adjusted with the addition of CAP more or less aversive than commercial levels of quantitative dietary restriction.To validate the study methodology, a second group of birds was given a choice between environments associated with *ad libitum*(AL) access to food or QFR. It was expected that the birds would show a preference for the *ad libitum* feed access environment. Novelty(preference for pen not housed in on the day of testing) was identified as a problem in previous work by Dixon *et al* (pers. comm). Therefore, here both groups of birds were tested twice (once on a day when fed QFR and known to be in a state of hunger and once on a day when fed the alternative diet option and in a state of satiety (*ad* *libitum* fed birds) or an unknown state (CAP-fed birds)). Thisenabledthe identification and of pen-novelty related effects. This also enabled the identification of any state-dependent preferences. It was predicted that state dependent effects would not be present as it was expected that birds would always prefer the environment that they associated with feeling satiated over one that they associated with feeling hungry.

3. Methods

3.1. Subjects

Twenty-four female Ross 308 broiler chicks were used from 28 day old birds. Broilers were used here as a more readily available model for parent stock. Prior to this study the birds had been group reared on a 14:10h light: dark schedule (day 1 – 28) and spot-brooded (day 1: 31˚C, reduced gradually to 21˚C on day 21 and maintained at this temperature thereafter). The birds were fed a commercial starter chick crumb (Farmgate, BOCM Pauls Ltd., Ipswich, Suffolk, UK)*ad libitum* from 1 – 14 days and, thereafter, feed restricted in line with the recommended daily feed requirements for broiler breeders (Aviagen, 2007). The mean (standard deviation) bodyweight of the birds on admittance to the study was 528.8g (± 32.3g) which was 20% heavier than the target bodyweight for broiler breeders at 28 days (440g). They had no previous experimental history.

A study timeline is provided to provide a brief overview of the experimental design and use of the experimental subjects (Table one).

TABLE 1 SHOULD GO HERE

3.2. Treatment groups

Birds were blocked according to weight, and then randomly allocated to one of two treatment groups on day 28. The treatment groups were: 1) QFR versus qualitative feed restriction (in which the food had calcium propionate added; QFR/CAP, n = 12), and 2) QFR versus *Ad libitum* feed (QFR/AL, n = 12). Each bird thus experienced two different diets, depending on treatment group. There were two phases to the experiment: 1) pre-CPP stage, and 2) CPP training and testing. The diet regime remained the same across both phases.

3.3. Diet and feeding regime

Irrespective of treatment, all birds were fed at 09:00h. Feed remaining at 17:00h was removed and weighed. Daily feed intake was recorded for each bird. All birds alternated every two days (from day

28 – 67 (end of study)) between the two diet options assigned to their treatment group. Within each treatment group, half the birds received QFR on the first two days followed by the alternative diet option (CAP or AL) on the following two days, and alternated thereafter (n = 6 per treatment). The other half of the birds received these diet options in the reverse order (n = 6 per treatment).

The basis of all the diets was a custom-made grower mash (Target Feeds, Whitchurch, Shropshire, UK) suitable for broiler breeders from 28 days of age. The diet was formulated to contain 165g/kg crude protein and 12.1MJ ME/kg feed. Birds in the treatment group QFR/AL received only this diet. They alternated every second day between QFR and *ad libitum* access to this diet between 09:00h – 17:00h. Birds in the treatment group QFR/CAP alternated between QFR and a diet that was qualitatively restricted by the inclusion of calcium propionate (CAP). The CAP option was the same quantity of diet as received under QFR *plus* the addition of calcium propionate (Propimpex® CA powder, Impextraco, Germany) mixed into the ration. The quantity of calcium propionate was increased over the duration of the study, from 30g – 90g/kg total feed (3 - 9%). The inclusion rate started at 3% based on previous work by the authors (Buckley *et al* 2011a) which indicated that this ration would be consumable within 8h. This was then increased to maximise time taken to consume ration whilst at the same time aiming to ensure that all birds fed that ration on that day had consumed ≥ 95% of the total ration by 17:00h. Thus, the calcium propionate level was increased to 4% on day 36 (or day 38), 5% on day 41 (43), 6% on day 45 (47), 7% on day 49 (51), 8% on day 57 (59), 9% on day 60 (62) and remained at 9% until the end of the study. The levels of CAP were increased based on previous work by the authors (unpublished observations) and Sandilands et al (2005) who found that it was necessary to increase the levels of calcium propionate included in the feed over time. This was presumably necessary as birds either adjusted to its properties or increased in relative severity of feed restriction. Birds were observed hourly between 09:00 – 17:00h and the hour by which the full ration was consumed was noted. At the end of the day any ration remaining was weighed: if ≥ 95% of the ration had been consumed the bird was considered to have consumed the full ration within 8h. The QFR ration was always fully consumed by 8h; however, the CAP ration was not (see figure four). During the pre-CPP phase (days 28 – 43), where the ration failed to be fully consumed by 17:00h it was left in the birds' pen overnight to allow additional time to consume the ration. On these days all birds remained individually housed overnight. During the CPP phase (days 44 – 67), surplus food was discarded at 17:00h for birds failing to meet the criterion. This applied to one bird on day 45 and three birds on day 51 with the mean daily quantity consumed (% of total ration) for these birds on these days was: 47.8g (90%) on day 45 and 48.6g (90%) on day 51.

3.4. Housing & husbandry

For the duration of the study, the birds were individually housed during the light hours in pens containing wood shavings, a perch and a drinker allowing *ad libitum* access to water. Birds were pair-housed overnight (exceptions outlined in the diet and feeding regime section).

The standard enclosure was a 0.95m (width) × 1.05m (length) × 0.8m (high) solid-sided metal pen. These pens were split down the middle with a wood divider creating two identical smaller pens measuring 0.475m × 1.05m. Each divider had a removable solid door (0.4m high × 0.25m long) set into the front bottom corner of the divider. The removable solid door was replaced with a removable mesh door for the first 10 days to reduce the initial stress of social isolation in the birds. The front of the pen comprised two sections. The top 0.4m was a full-length mesh door that could be opened to allow easy access to the pen. The bottom 0.40m was a full length solid wooden divider. Set midway along this divider was a hatchway (0.25m × 0.25m) with a guillotine door. This hatchway lined up with the central pen divider such that the divider bisected the guillotine door.

There were 12 of these divided pens in total. One bird was housed on each side of the divider (i.e. in one of the 24 smaller pens).The feed bowls were placed at the front of the pen attached to a mesh grid. The distance between the feed bowls and the floor was adjusted as periodically as the birds grew to ensure ease of access but minimise spillage of feed.

The birds' pens werewithin a room that was maintained at 21˚C throughout the study. The photoperiod was gradually reduced from 14h / day (day 28) to 9h / day (day 33) to ensure the birds experienced a similar light period as commercially reared broiler breeder birds who are maintained on 8h light (it was slightly longer in our study to account for end of day experimental procedures (e.g. feed removal, etc). Thereafter, birds were given 9 hours light / day (09:00h – 18:00h) with the exception of the 6days on which pre-existing side bias testing and CPP testing occurred (days 37, 38, 39, 40, 55 and 67). On these days the number of hours of lighting was extended until 21:00h to allow the end of day data collection to occur.

3.5. Pre-CPP phase

The purpose of the pre-CPP phase was to allow the birds to habituate to the test conditions (solitary housing), to adjust to the diet options on offer (to prevent dietary neophobia or initial sensory-led preferences affecting CPP formation) and to enable the identification of any birds with side-biases to allow this to be controlled for.

3.5.1. Housing protocol during pre-CPP phase

The pre-CPP phase took place between days 28 – 43. Birds were housed individually in the divided pens between 09:00h – 17:00h. At 17:00 the door in the central divider was removed allowing the birds housed on each side of the divider to move freely between the two pens. The two birds were allowed to interact until 18:00h before being returned to their pen(if any birds had failed to fully consume the feed ration) or 09:00h (if all birds had fully consumed the feed ration). All birds were housed individually overnight if any had not fully consumed the ration in order to maintain consistency between birds. This social interaction was instigated on ethical and welfare grounds and no data was collected during this interaction. In practice, there were nine days during the pre-CPP phase in which it was necessary to house the birds individually overnight.Birds alternated daily between the two pens they were housed in to habituate them to regular changes of environment whilst preventing the association of the diet options with specific pens during the pre-CPP phase, since diets alternated every two days throughout.

3.5.2. Side bias testing during pre-CPP phase

Each bird was tested 4 times for the presence of a pre-existing side bias. Side bias testing took place between 17:15h–20:15h on days 37, 38, 39 and 40. Testing was balanced within bird with half the tests taking place on days when the bird had been fed QFR and half the tests taking place on days when the bird had been fed the alternative diet option (CAP or AL). Half the tests took place on the 1st day post switch to the QFR (or alternative diet option) and the remaining half took place one 2nd day (i.e. the day before switching to the other diet option). The side-bias testing procedure was as follows: each bird was removed from its pen 15 – 20 minutes before it was tested for a side bias and placed in a holding pen. The bird occupying the adjacent pen was removed and placed in another holding pen just before testing of the first bird commenced. Both pens (each side of the divider)were cleaned out and fresh wood shavings added. The feed bowl and associated attachments were removed. The door in the wooden divider was removed. The bird was then placed in a box (0.25m (w) x 0.30m (l) x 0.35m (h)) that was lined up with the guillotine door. After 30 seconds the guillotine door was raised and the bird was allowed to enter either pen and allowed to move freely between both pens for 20 minutes. The bird was then removed, returned to the holding pen and the other bird tested. Once both birds occupying adjacent pens had been tested both birds were returned to the pens. First penentered was recorded. Each bird was observed continuously and each time the bird changed pens this was recorded in seconds using a stopwatch. A bird was considered to have changed pens when both feet had entered the neighbouring pen.

The criterion for a bird being considered to have a pre-existing side bias was more than 60% spent on a particular side out of the total amount of time the bird was observed for (80 minutes). This 60% threshold was based on the work of Dixon *et al* (pers. comm.). Within the QFR/AL group 4 birds had a right sided bias and 5 birds had a left sided bias. Within the QFR/CAP group 6 birds had a right-sided bias and no birds had a left-sided bias.

3.6. CPP phase

This phase comprised CPP training and both tests for the existence of a CPP.

3.6.1. Conditioned place preference apparatus

The CPP apparatus was the same divided pens as used for the pre-CPP phase as described above butcoveredfrom floor level to a height of 0.7m on the side and back walls of the pen with sheets of varnished and laminated paper. Each adjacent pen (i.e. separated from each other by the divider) had paper sheets with one of two patterns. In one pen the pattern was vertical black and white stripes(33mm wide stripes),while in the other pen it was horizontal black and white stripes (16mm wide stripes; Figure 1). This was balanced such that half the 'vertical-striped' pens were on the right side of the divider and the 'horizontal-striped' pens on the left side and vice versa. Both pens were designed to ensure an equal coverage of black and white to control for brightness.

FIGURE 1 GOES HERE: DIAGRAM OF CPP APPARATUS

3.6.2. CPP training and housing protocol

On day 44, CPP training began and lasted until day 67 (end of the study). Each bird lived in one of the distinctive pens on days when it received QFR and the other distinctive pen on days when it received AL (QFR/AL treatment group) or CAP (QFR/CAP treatment group). The aim was to allow the birds to associate the different pens with the state of hunger that they experienced within them. Within treatment, half the birds (n = 6) experienced QFR in the vertically striped pens (for three birds this was the right sided pen and for three birds this was the left-sided pen) and half (n = 6) experienced QFR on the horizontally striped pens (also balanced for pen side). To control for pre-existing side biases birds that demonstrated a pre-existing side bias were approximately equally distributed such that, within each treatment group, half the birds received QFR on their preferred side and half received QFR on their least preferred side.

Birds lived in the CPP apparatus continuously. Between 09:00h – 17:00h birds were housed individually. Overnight, they were pair-housed with another bird. The conspecific they were housed with remained the same throughout this phase. Birds were paired according to treatment group and pen pattern experienced that day (i.e. a bird that had been housed in a vertical striped pen was housed overnight with another bird that had experienced vertical stripes that day and both birds were from the same treatment group). On days when birds were switched between environments they were switched at 09:00h before being fed.

3.6.3. CPP testing

Each bird was tested for a CPP after 12 days training (6 days per diet: environment pairing; tested on day 55) and 24 days training (12 days per diet: environment pairing; tested on day 67). The CPP testing protocol was identical to theprotocol for side bias determination.

3.7. Statistical analysis

All statistical analyses were undertaken using Genstat (13th Edition, VSN International, Ltd., Hemel Hempstead, UK). The Shapiro-Wilks test was used to evaluate the distribution of proportion data which was normal. Therefore, untransformed data and a repeated measures REML was used to investigate any differences between treatments, effect of state at the time of testing and interactions between these. Number of pen changes was normalised using the log-e transformation prior to REML analysis. For all analyses, subject was the bird ID. The relevant time point was test number and these were equally spaced and identical between subjects. The fixed effects were: treatment, diet option fed on day of testing, pattern associated with QFR and interactions between these. The variables of interest tested were: proportion of time spent on the non-QFR side; proportion of time spent in the 'novel' pen; proportion of time spent in the right pen. Differences from 0.5 were evaluated using the One-sample T-test. Where confidence intervals are reported these are at the 95% significance level.

First pen entered data was tested using a GLMM with a logit-transformed binomial distribution. The variables of interest included: which pen was entered (associated with QFR or alternative diet; right or left sided pen; novel pen or pen bird spent the day of testing in). The fixed effects examined included: treatment, state at time of testing; pattern associated with QFR and all interactions between these. The random effect was bird ID. Differences from 0.5 were calculated by chi-square (1 d.f.) using a test statistic generated by the following formula: χ^2 = (predicted mean / S.E. of the predicted mean)^2. Predicted means were generated by GLMM.

3.8. Ethical considerations

This study was carried out under Home Office license and was approved by both the Scottish Agricultural College's and Roslin Institute's Animal Ethics Committees. Pen sizes exceeded the minimum recommendation for individually housed poultry and shavings and a perch were provided to facilitate natural behaviour. Due to the study design, it was considered necessary to house birds individually during the day; however, birds were pair-housed overnight (17:00 – 09:00h) for the majority of the study to allow for some social interaction as it is recognised that chickens are a social species. Feed restriction was no more severe than under commercial conditions (and, in the case of the QFR/AL birds far less severe). All birds remained healthy during the study. At 93 days of age birds were sent for a post-mortem to assess any potential gastrointestinal tract pathology as a possible consequence of calcium propionate ingestion. No treatment-related pathology was identified.

4. Results

4.1. Growth curves

QFR/CAP birds grew at a similar rate to the commercial target (Figure 2), while QFR birds grew at a faster rate. This was to be expected as QFR/CAP birds were fed a similar quantity of basal diet as birds fed to commercial levels of feed restriction, while QFR/AL birds consumed considerably more feed on days when they were fed an *ad libitum* ration (Figure 3). The average consumption (± standard deviation) on *ad libitum* days was 58.3g (± 5.2g) (day 29) – 204.9g (±10.9g) (day 67).

FIGURE 2 GOES HERE: DIAGRAM OF GROWTH CURVES

Over the duration of training and testing the level of feed restriction of QFR/CAP birds was 22 - 24% (compared with birds of the same age) or 43 - 44% (compared to birds of similar bodyweight) of the QFR/AL birds *ad libitum* intake (range established from the first and last days of CPP training and resting, i.e. days 44 and 67 and based on the difference between the QFR ration for the QFR/CAP birds and the estimated daily intake of *ad libitum* fed broilers (Aviagen, 2007) for birds of a similar weight (age or bodyweight-matched respectively)).

FIGURE 3 GOES HERE: DIAGRAM OF FEED INTAKE

4.2. Time taken to consume QFR or CAP ration by QFR/CAP birds

As expected the CAP ration always took longer to consume than the QFR ration (Figure 3). However, there was considerable variation between birds in relation to the time taken to consume the CAP ration. The inter-day median time (with inter-quartile range shown) taken to consume the CAP ration across the period of CPP training and testing (day 44 – 67) was 6.2 (5.7 – 7.2) hours. However, intra-day there was considerable more variation apparent between birds (Figure 4).

FIGURE 4 GOES HERE: TIME TAKEN TO CONSUME EITHER THE CAP OR THE QFR RATION

4.3. Proportion of time spent in each pen

The main variable of interest was the proportion of time the birds spent in either the pen associated with QFR or the pen associated with *ad libitum* access to feed (QFR/AL treatment group) or CAP (QFR/CAP treatment group). Here an effect of state at the time of testing ($F_{1,16}$= 5.43, P = 0.033) was observed. Post-hoc testing from 0.5 (no preference) indicated that only hungry (i.e. fed QFR on the day of testing) QFR/AL birds showed a significant preference (T_{11}=3.27, P = 0.007). This preference was for the pen associated with *ad libitum* access to feed (mean preference: 0.653; C.I. 0.550 – 0.757) (Figure 5). Ten out of 12 QFR/AL birds spent more time (shown as a proportion of total time tested) on the non-QFR pen when tested under conditions of feed restriction. The QFR/AL group mean of 0.45 (C.I. 0.29 – 0.60) did not differ significantly from 0.5 on days when QFR/AL birds were satiated (T_{11}=0.75, P = 0.467). No significant pen preferences were observed for QFR/CAP birds either when tested on QFR days (T_{11}=1.19, P = 0.259) or on CAP days (T_{11}=0.52, P = 0.611). The QFR/CAP group mean was 0.57 (C.I. 0.44 – 0.69) on QFR days and 0.52 (C.I. 0.42 – 0.62) on CAP days.

FIGURE 5 GOES HERE: STATE*TREATMENT

No significant differences were found with pattern ($F_{1,16}$=2.3, P = 0.149) or side ($F_{1,16}$=0, P = 0.977)associated with QFR or test number (i.e. when tested after 12 and 24 days of training) ($F_{1,22}$=0, P = 0.946) in terms of proportion of time spent in each pen. Importantly, there was no effect of pennovelty with QFR/CAP birds showing no preference for either for or against the pen they had spent the last two days living inregardless of diet option fed on the day of testing (Tested on CAP day: mean: 0.48; C.I. 0.38 – 0.58; T_{11}=-0.34, P = 0.739; Tested on QFR day: mean 0.57; C.I. 0.44 – 0.69; T_{11}=1.19, P = 0.259). QFR/AL birds tested under conditions of hunger (i.e. fed QFR on the day of testing) showed a significant preference for the novel pen. In this instance this corresponded with the preference for the pen associated with *ad libitum* access to feed, suggesting that the treatment effect was responsible, rather than a preference for novelty under only these circumstances. They did not show a preference when tested on days when fed *ad libitum* (mean: 0.45; C.I. 0.40 – 0.7; T_{11}=0.75, P = 0.467).

4.4. First pen entered

First pen entered did not reveal any significant preferences for either distinct environment. There was no effect of treatment ($F_{1,22}$=0, P = 1), state at time of testing ($F_{1,42}$=0.33, P = 0.566) or pattern ($F_{1,21.9}$=2.75, P = 0.111). Neither group entered the pen associated with novelty (QFR/AL: χ^2=0.24, d.f. = 1, P > 0.05; QFR/CAP: χ^2=0.16, d.f. = 1; p > 0.05)or the pen associated with the non-QFR diet option (QFR/AL: χ^2= 0.10, P > 0.5; QFR/CAP: χ^2=0.10, P > 0.05) significantly more or less than 0.5. Furthermore, a side bias was no-longer evident in either treatment group (QFR/AL: χ^2=0.10, P > 0.05; QFR/CAP: χ^2=2.60, P > 0.05).

4.5. Number of pen changes

The median (inter-quartile range) number of changes between pens during tests was 14 (9 – 19.5) for the QFR/AL treatment group and 18 (11 – 25.5) for the QFR/CAP treatment group which was not statistically significant ($F_{1,22}$=2.47, P = 0.13).There was also no effect of diet option fed on day of testing ($F_{1,22}$=0.2, P = 0.661) and no interaction between treatment and diet option fed on day of testing ($F_{1,22}$=0.07, P = 0.793).

5. Discussion

The key significant findings from this study were that the QFR/AL birds expressed a preference for the pen associated with *ad libitum* feeding but only under conditions of deprivation (hunger) and the birds in the QFR/CAP group failed to demonstrate a preference for the environment associated with either diet option.

5.1. The QFR/AL birds' pen preferences

The finding that feed restricted broilers could learn a food-rewarded CPP under certain circumstances (the control group, i.e. QFR *vs.* AL feeding) but express it only when acutely feed restricted was unexpected. The failure of the QFR/CAP birds to show evidence of attraction to the novel pen suggested that the QFR/AL birds were not attracted to a pen due to its relative 'novelty'. Further, the lack of difference in pen changes between the two groups of birds or interaction with state at time of testing provided a crude indicator that the QFR/AL birds when tested under conditions of hunger had not simply picked a pen to forage in(anecdotally, the predominant activity) and then failed to move. Rather, theyrepeatedly returned to their favoured side. Thus, it seems that a state-dependent preference was being observed. This provided an interesting additional or alternative explanation for the birds' preference expression. It had been assumed that the birds would pick the pen associated with feeling more satiated because this is a positive affective state and birds would prefer to spend their time in a pen they associate with feeling 'good' (satiated) rather than in a pen they associate with feeling 'bad' (hungry). This is the basis for most CPP tests in pharmacological research (Tzschentke 1998; Bardo and Bevins, 2000). However, Spiteri*et al* (2000) found that morphine-conditioned rats spent less time active and more time in close association with the conditioned stimulus. By comparison, food-conditioned rats were more active and showed more exploratory behaviour. They concluded that rats given morphine had associated the environment with the post-affective state induced by morphine. By contrast, rats rewarded with food had learnt that the food-rewarded environment was a good place to find food which stimulated appetitive, food-seeking behaviour. This suggests that the QFR/AL birds in our study, when tested on days when hungry, perhaps selected the pen associated with *ad libitum* food supply not because they associated that environment with a more positive state but because they anticipated that they would be more likely to obtain food within this pen.

Few food rewarded-CPP studies have used a within-subject comparison between state of deprivation (e.g. hungry versus not hungry) to assess hunger-state-dependent preferences. Perks and Clifton (1997) trained food deprived (to a bodyweight no less than 85% of *ad libitum* intake), water-satiated rats to associate one environment with sweetened mash diet and another environment with sweetened water. Both rats were then tested under two different motivational states: thirst and hunger. They found a state-dependent preference: the rats preferred the pen associated with water when thirsty and *vice versa*. This indicated that therats associated each distinct environment with resources of potential future value rather than post-consummatory affective state during training (although this latter association may also have occurred). Otherwise, the rats would have shown a preference for the mash-associated pen as they encountered this in a state of deprivation during training so its motivational value at the time of learning should have been higher than the sugar water. Further, the authors demonstrated that devaluing the sugar water post training of the CPP by pairing it with lithium chloride (in the home pen) reduces the strength of CPP expressed.

Where between-subject studies have been performed they have indicated that the pre-fed animal demonstrates either no CPP (Figlewicz*et al* 2001) or an attenuated CPP (Bechara and van der Kooy, 1992; Lepore*et al* 1995). Although some studies have found a food-rewarded CPP in non-deprived subjects (Papp, 1988; Papp, 1989; Papp *et al* 1991; Bechara and van der Kooy, 1992; Muscat *et al* 1992; Willner, *et al*, 1994; Lepore*et al* 1995) the studies by Papp (1988, 1989, 1991), Muscat (1992) and Willner (1994)all adopted a methodology that included feed restriction throughout training. The rats are described as pre-fed before training but limited detail is available so it is difficult to determine how satiated the rats would have been before testing for CPP. It seems unlikely that the rats would have fully compensated for chronic feed restriction during training during the small interval between cessation of training and the CPP test (at most 24 hours). By contrast, our methodology in which broilers alternated every two days between feed restriction and *ad libitum* feed regimes probably allowed the birds to compensate to a degree as broilers can increase feed intake to near *ad libitum* levels on skip-a-day regimes (Dunnington, 1987). Thus our QFR/AL birds were expected to be satiated on days when given *ad libitum* access to feed prior to CPP testing.

Most food-rewarded CPP tasks train and test the animals under the same condition (Feed restriction: Guyon, *et al* 1993; Popik and Danysz, 1997; Chaperon, *et al* 1998; Spiteri*et al* 2000; Figlewicz*et al* 2001; Yonghui*et al* 2006; Zombeck*et al* 2008; Koizumi, *et al* 2009; *Ad libitum* access: Imaizumi*et al* 2000, 2001; Jarosz*et al* 2006; Dickson *et al* 2010; Matsumura *et al* 2010); therefore, it is not possible to clearly disentangle the effects of training (state-dependent learning) from testing (state-dependent preference). However, state-dependent preferences have been observed in food- and sucrose water-rewarded CPP tests. Naloxone (a dopamine receptor antagonist) (Jarosz*et al* 2006) and Naltrexone (an opioid receptor antagonist) (Delamater *et al* 2000) abolish food and sucrose conditioned CPPs respectively when injected before testing rats for CPP presence. In contrast, the dopamine receptor agonist, MK-801, both increases feed intake and potentiates expression of food-rewarded CPPs when administered pre-test (Yonghui*et al* 2006). Finally, Larson (2006) found a sucrose-water CPP was only expressed when rats were water deprived prior to testing. These studies indicate that state at time of testing can affect the expression of food (or water) rewarded CPP. The current study supports these studies and indicates that the state of the animal during testing should be considered when designing CPP studies to determine feed preferences in feed restricted broilers.

However, environmental preferences have been observed in animals pre-fed prior to testing for a food-rewarded CPP. Papp (1988), Papp *et al* (1990) and Spyraki*et al* (1982) all trained under deprivation and fed prior to testing and found the rats demonstrated a CPP (but the effects of prior deprivation cannot be discounted as a motivator in these methodologies). No studies were found that trained under *ad libitum* conditions and tested under conditions of feed restriction (i.e. tested during a state of deprivation). Imaizumi*et al* (2000, 2001), Jarosz*et al* (2006), Matsumura *et al* (2010) and Dickson *et al* (2010) used rats fed *ad libitum* on chow outside the training situation. However, they trained a CPP in which the rewarding environment was associated with a higher value 'treat' food not available outside of the test situation. For example, corn oil (Imaizumi*et al* 2000, 2001) high sugar or high fat foods (Jarosz*et al* 2006), chocolate drops(Dickson *et al* 2010) or pre-training gastric infusions of glucose or corn oil paired with low nutritive foods within the apparatus (Matsumura *et al* 2010).By contrast, the less rewarding environment was associated with rat chow (except Imaizumi*et al* 2000, 2001, who used plain water). However, it is reasonable to assume any CPP that develops under these conditions develops as a consequence of a hedonic state induced by something other than the reduction of hunger. Thus, attainment of satiety (or, at least, reduction in hunger) is not a necessary condition of food – rewarded CPP learning. This has implications for the use of CPP to determine affective state in quantitative or qualitatively-restricted broilers both in how the test should be used and what should be inferred from the demonstration of a food-rewarded CPP.

5.2. QFR/CAP birds failure to show a preference

The state-dependent preference observed in the QFR/AL group does not explain why the birds in the QFR/CAP group did not express a preference for one of the distinctive environments. This could have resulted from a failure to express a preference despite having learnt the relevant associations with environment or a failure to learn the task (and thus an inability to express any preference). These shall be discussed in turn.

5.2.1. *Learnt the task but no preference exhibited?*

One possibility for the failure to exhibit a preference is that the birds genuinely did not have a preference for either environment, perhaps because both distinct environments provided similar opportunities for the reduction of hunger. Alternatively, whilst differing across various dimensions (e.g. post-ingestion effects, sensory-led effects) the net effect in terms of affective state for the bird may have been perceived as similar between environments (e.g. the QFR environment may have offered a more palatable diet option than the CAP environment but resulted in higher levels of hunger than that experienced in the CAP environment). This study was not designed to investigate foraging decisions in hungry broilers. However, the finding that the QFR/AL birds expressed a preference for the pen associated with *ad libitum* feed access suggested that the birds selected the pen based on whether it was previously a good environment to forage in. Therefore, if a broiler's foraging behaviour is sensitive to time and it is able to recognise when food is likely to be available within an environment then our study design contained an inherent weakness. Namely, birds were tested during a period in which they had never received, or had access to, food. Most food-rewarded CPP studies are not closed economy and are likely to conduct their tests during a similar time of day to which the training took place. Therefore, the animal would enter the CPP apparatus expecting to find food within the chamber(s) that it had associated with food.

Both previous research by the authors and anecdotal observations in the current study suggested that the CAP option was aversive. Tolkamp*et al* (2005) noted oral lesions (presumably associated with pain)when feed restricted broiler breeders were fed a mash diet which included 90g calcium propionate/ kg total feed. In the current study no gastrointestinal lesions were noted either during the study or at post-mortem. However, it was informally observed that some birds tried to escape the pen immediately upon being given their CAP ration (but never their QFR ration). This suggested that, whilst CAP was not associated with lesions (and the associated discomfort) it was not as favourably received by the birds as the QFR ration. Thus, it was unexpected that a preference would not be observed during the formal testing and suggested that the diet option: distinct environment association had not been learnt.

5.2.2. *A failure to learn the task?*

A failure to learn the task appears counter intuitive given that QFR/AL birds did learn the task. However, several points can be made in favour of this interpretation. Firstly, QFR/AL birds expressed this state-dependent preference when hungry. There was *at least* one diet condition under which the QFR/CAP birds would have been hungry (QFR days). Therefore, there was *at least* one day during which the birds would have been in a state in which 'preference expression' (assuming one existed) could be anticipated. If birds were hungry on only one day or, at least *less hungry* on one day this would suggest that one diet was more satiating (and, presumably, more rewarding) and should have been preferred. Despite this, a preference was not expressed.

Secondly, ifthe effects of CAP on bird wellbeing (positive or negative) are not due to increased satiety, then the birds were trained and tested while fed a quantity of feed similar to commercial levels of feed restriction.Quantitative feed restriction is associated with behavioural and physiological indicators of stress in broiler breeders (e.g. Hocking *et al* 1993, 1996; de Jong *et al* 2002, 2003). Feed restriction is also associated with physiological changes such expression of, and levels of, certain nutritional-status-related compounds that may affect cognition (e.g. ghrelin, Diano*et al* 2006; synapsin proteins, Deng *et al* 2009). Buckley *et al* (2011b) found feed restriction resulted in poorer performance on a food quantity discrimination task with all birds fed to commercial levels of feed restriction failing to learn a food quantity discrimination task. Although most animals taught a food-rewarded conditioned place preference task are feed restricted, the level of restriction is less severe than the birds experienced (assuming that CAP had no or minimal satiating effects). Where reported, most studies restricted their animals (rats or mice) to somewhere within the range of 80 – 90% of *ad libitum fed* bodyweight (85 – 90%, Lepore*et al* 1995; 85%, Delamater *et al* 2000; 90%, Stuber et al, 2002; 80 – 85%,Yonghui*et al* 2006) or circa 50% of expected *ad libitum* intake (Figlewicz*et al* 2001). This was less severe than the birds in this study. Further, for these animals, feed restriction began shortly before the study commenced. By contrast, the birds in the current study had experienced feed restriction from 14 days of age.Therefore, the birds in this study were considerably more feed restricted than in most other studies and this may have negatively affected learning.

Thirdly, high doses of propionate (sufficient to induce acidaemia) have been associated with later learning impairments in rats (Brusque, *et al* 1999; Pettenuzzo, *et al* 2002; Shultz, *et al* 2009; MacFabe, *et al* 2011). However, methodological differences limit the inferences that can be drawn. For example, those studies administered propionate subcutaneously (Brusque *et al*, 1999; Pettenuzzo, *et al* 2002) or via intracerebroventricular injection (Schultz, *et* al 2009; MacFabe, *et al* 2011) whereas the birds in our study received CAP orally and could choose how much they ingested and over what timeframe. Despite this, it cannot be discounted as a possible factor affecting the ability of the QFR/CAP birds to learn the CPP task.

Finally, extraneous stressors may have synergistically interacted with dietary stressors to prevent learning in the QFR/CAP birds. Chickens are a social species so social isolation can be expected to be stressful. Chronic social isolation negatively affected morphine or heroin rewarded CPP formation in rats (Kiyatkin and Belyi, 1991; Courdereau, *et al* 1997).The birds in the current study were individually housed during the day during the training and testing periods.This methodology was adopted due to concerns that testing the birds in pairs contributed to the lack of preferences exhibited in the Dixon *et al* (pers. com) study. However, the long latency to consume the QFR ration by 4 week old QFR/CAP birds (data for QFR/AL birds was not recorded) immediately post-separation was atypical and unanticipated (they were consuming the daily ration in less than 40 minutes (unrecorded data) in the couple of days immediately preceding separation). The most reasonable explanation is this was primarily the effect of separation as increased vocalisation and attempts to access the other bird were evident. Further, the switch between the QFR ration and the CAP ration may have been experienced both as an uncontrollable and unpredictable environment condition (key components of many stressors, Wiepkema and Koolhaas, 1993) which would act as additional stressor. Exposure to chronic low level stressors has been demonstrated to abolish or attenuate either the learning and / or expression of a food – rewarded CPP task (Papp *et al* 1991; Cheeta*et al* 1994; Willner*et al* 1994). This may be particularly relevant in studies that use a closed economy design as the impact of environmental stressors can be protracted during CPP training.

5.3. <u>Other methodological issues</u>

In theory, the birds were tested during extinction (absence of food and food bowls). In practice, these may not have been true extinction conditions. During testing, the pens contained wood shavings. Informal observations made during this and other experiments by the authors (unpublished observations) and Dixon (pers. comm.) indicate that the birds utilise these shavings extensively for foraging. It is inevitable that spilt food will be discovered reinforcing this behaviour. Further, in other experiments by the authors, birds consume wood shavings with considerable crop fill noted for some birds both whilst alive (author's own observations) and during post-mortem (Hocking, pers. comm.).

Thus, shavings may have non-nutritive satiety-promotingproperties (assuming that a full crop promotes satiety). In addition, shavings allow some natural behaviour to occur, occasionally yieldinga nutritive morsel and distracting the birds' attention from the cues signifying the diet option to be found within this environment. Regardless of the underlying potential value or impact of providing shavings it is suggested that it was an error to provide (or at least not control for) shavings during testing. De Jong *et al* (2008) investigated CPP formation in pigs and found that pigs could form a CPP to an environment containing straw to forage in suggesting this was rewarding to pigs. Despite this, the performance of the QFR/AL birds indicates that, even with shavings provided during testing birds are able to demonstrate a CPP. This does not, however, account for the expected differential and / or relative value of shavings under the various feed options the birds encountered.

6. Conclusions and further research

It is concluded that there is some evidence that feed restricted broilers can learn a food quantity associated CPP task. However, the presence of state-dependent preference expression means that it is essential to take this into consideration when designing such studies to maximise the chances of identifying a preference where one exists. Further, there was no evidence that CAP improves the welfare of feed restricted birds. Whilst a CPP was not observed, informal observations indicated that the birds did not like the CAP diet. Therefore, the more plausible interpretation is the birds failed to learn the task.However, this has not been shown by this current study and a genuine lack of preference cannot be discounted. Thus, further research should investigate the effect of both plane of hunger and calcium propionate on ability to learn a CPP task before adopting this technique more widely as a tool for the assessment of the welfare benefits of qualitatively-restricted diets.

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Table 1: Study timeline showing key information relating to the experimental design

Day	0 - 27	28	28 - 43	44 - 67	67
Phase	Pre-study	Start CPP experiment Allocate birds to treatment groups	Pre-CPP	CPP	End CPP experiment Birds maintained on the same every other day alternating diet option schedule Day 93: Euthanasia and post-mortem of all birds
Housing	Group - housed		Day: individually housed Night: group housed		
Treatment groups	N/a		1. QFR/CAP (n = 12) 2. QFR/AL (n = 12)		
Diet options	All: commercial starter pellet		1. Mash grower diet ±calcium propionate for QFR/CAP birds 2. Mash grower diet - feed restriction or ad libitum access (QFR/AL birds)		
Diet protocol	Fed AM, feed restricted from 15 days		All birds: Alternate **every second days** between QFR and alternative diet option i.e. AA, BB, AA, BB, AA, …		
Pen design	N/a		Plain walls	Vertical and horizontal black and white striped walls	
Pen protocol	N/a		Alternate **every other day** between right and left pen i.e. A,B,A,B,A, …	Alternate **every second day** between the right and left pen (in line with diet option switches) i.e. AA,BB,AA,BB, …	
Test protocol	N/a		All birds: 4 side bias tests: once per bird on days 37, 38, 39 & 40	All birds: 2 CPP tests: Once per bird on days 55 & 67	
Diet option fed on day of test	N/a		All birds: 2 tests per bird on days when fed QFR; 2 tests each per bird on days when fed the other diet option	All birds: 1 test per bird on a day when fed QFR; and 1 test per bird on a day when fed the other diet option	

Figure 1: Diagram of CPP apparatus (not drawn to scale). The front of the pen (demarked by the two horizontal stand-alone lines) has been cut away to allow the reader to better visual the pen set-up during CPP testing.

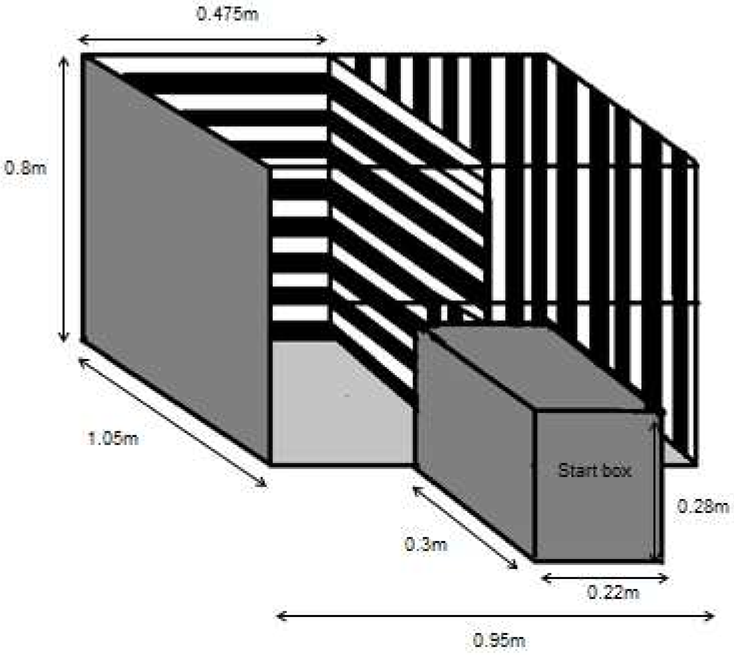

Figure 2: The growth rate of the birds in treatment groups QFR/AL and QFR/CAP. The target growth rate for Ross 308 Broiler Breeders (fed to 5% production at 25 weeks) is also shown for comparison. Error bars are omitted as the S.E.M. for each group was too small to illustrate effectively. The S.E.M. for each of the time points shown was as follows: QFR/AL: 9.1g; 12.2g; 23.5g; 26.2g; 37.2g; 32.5g; 43.0g; QFR/CAP: 9.9g; 6.7g; 6.7g; 7.9g; 9.0g; 7.9g; 9.0g. Bird growth rate is shown to 70 days (this trial ended on day 67 but the birds remained on the same diet protocol as they were re-used for a further experiment).

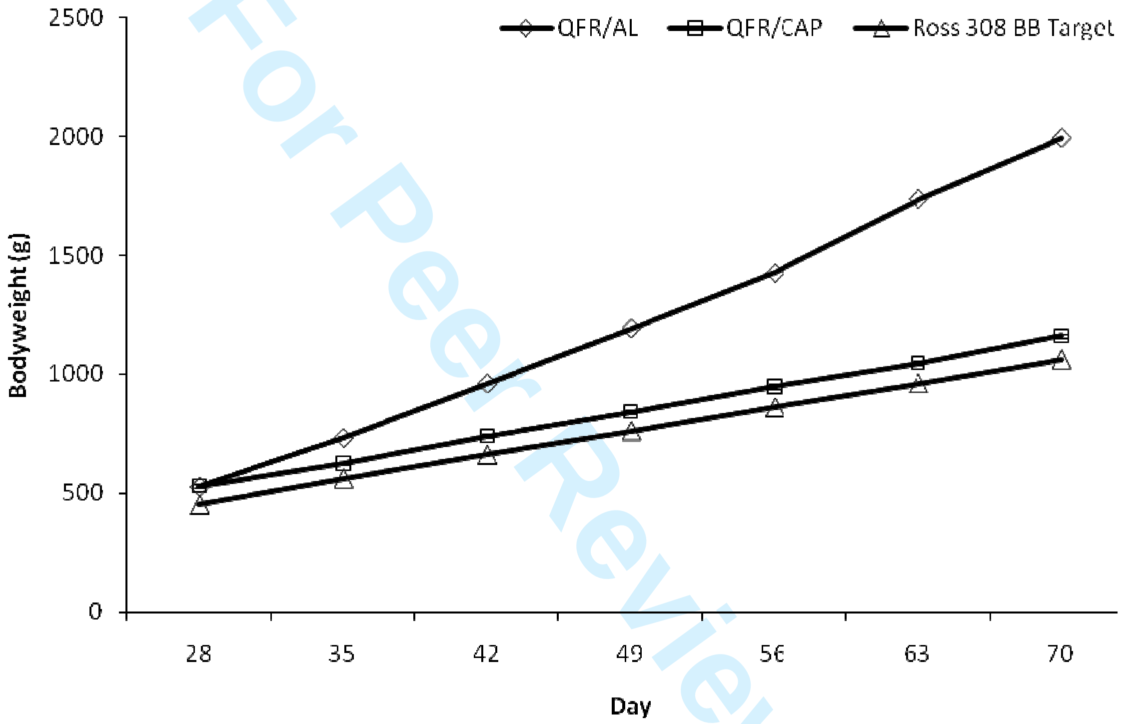

Figure 3: Daily feed intake of the diet options by the treatment group QFR/AL and QFR/CAP. The error bars indicate the daily S.E.M. and are shown only for the AL group. The mean daily S.E.M. for QFR intake for both the QFR/AL and QFR/CAP treatment groups was 0g. The mean daily S.E.M. for CAP intake was 1g.

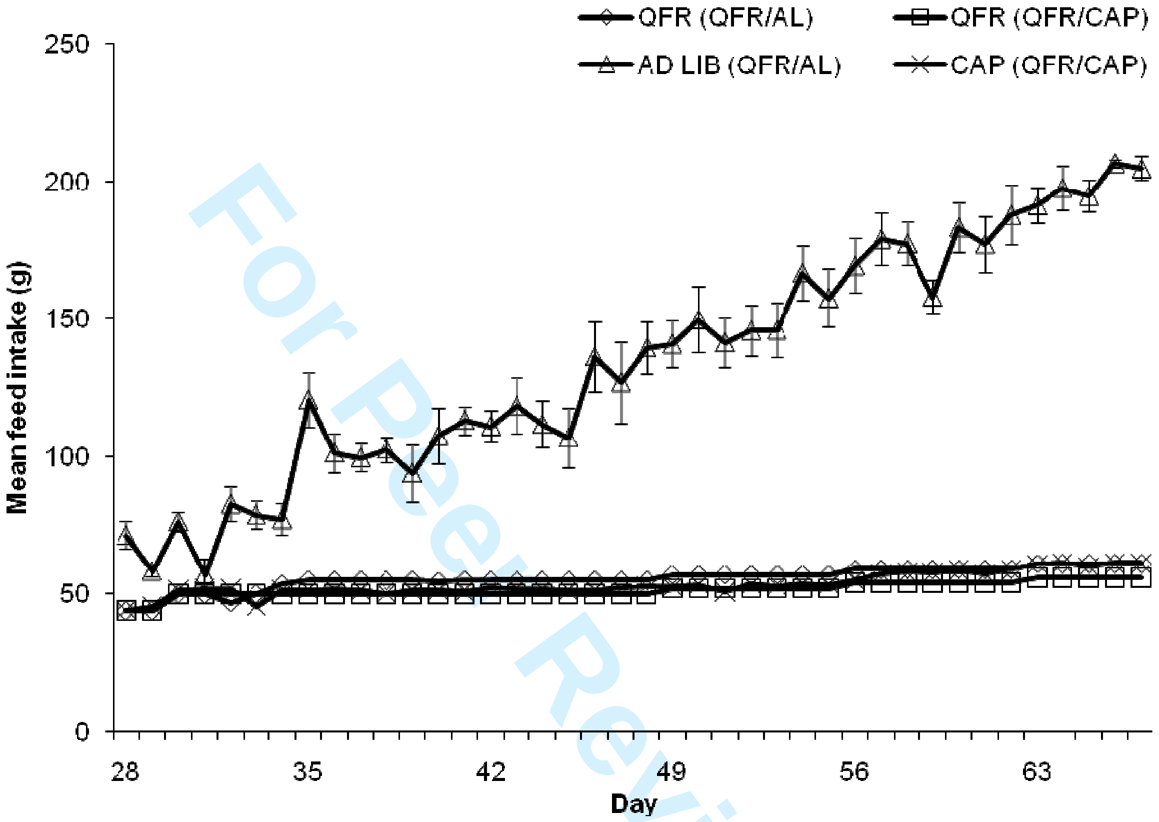

Figure 4: The time taken to consume either the CAP or the QFR ration by the treatment group QFR/CAP. The Inter-quartile ranges are shown by the error bars. The dashed horizontal line represents the 8h cut off point. Birds failing to consume ≥ 95% of the daily ration by the 8h cut off were awarded 9h as a nominal value to aid graphical representation. There were no days on which birds failed to fully consume the QFR ration by 8h. The number of birds that failed to consume the full CAP ration by 8h are as follows: day 28: 3; day 29: 5; day 30: 5; day 31: 5; day 32: 3; day 33; day 37: 1; day 38: 1; day 41: 2; day 45: 1; day 51: 3; on all other days 0. The calcium propionate inclusion rate started at 3% and was increased to 4% on day 36 (38), 5% on day 41(43), 6% on day 45 (47), 7% on day 49 (51), 8% on day 57 (59), 9% on day 60 (62) and remained at 9% until the end of the study (day 67). Data was unavailable for CAP consumption on day 53.

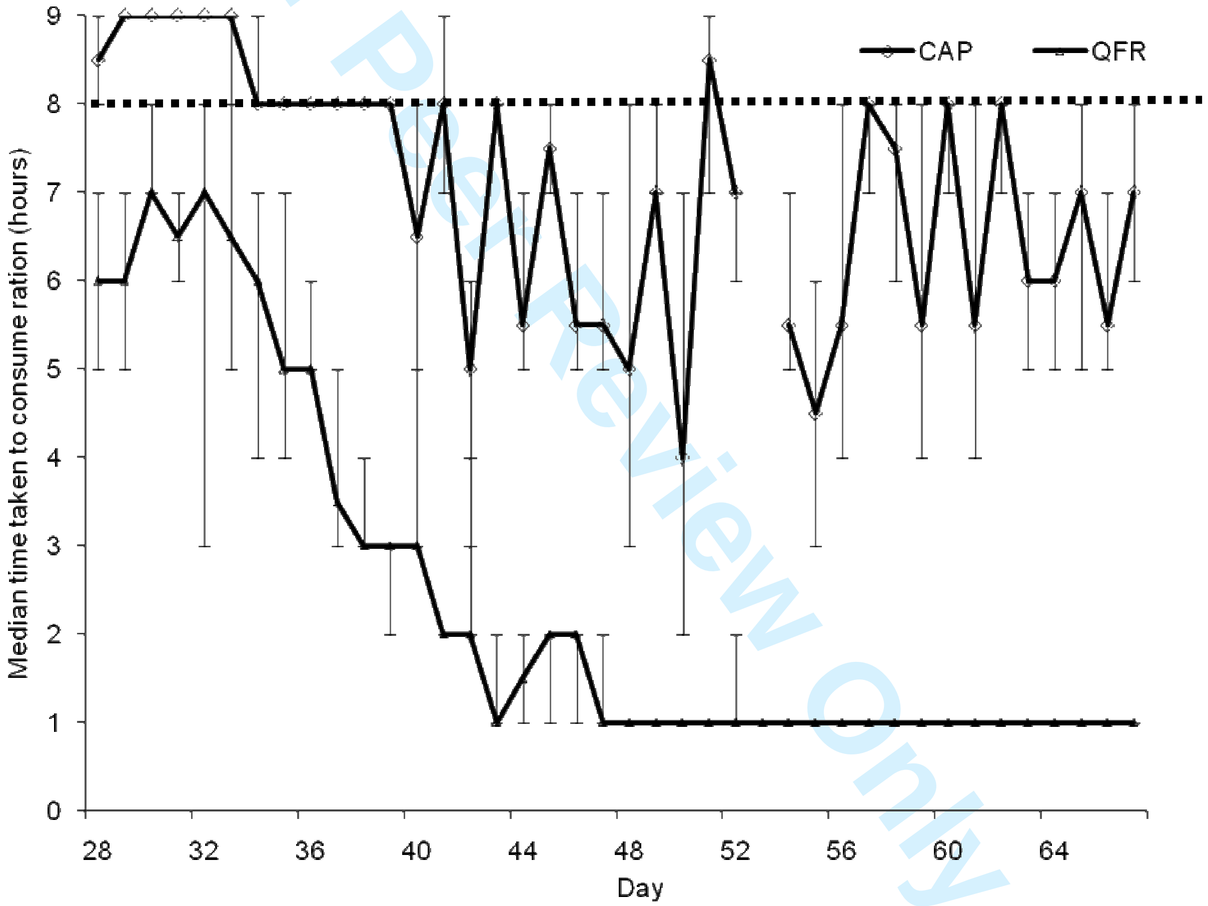

Figure 5: Effect of treatment and state at time of testing on proportion of time spent in the non-QFR associated pen. S.E.M. is indicated by the error bars. Only the QFR/AL treatment group, tested in a state of hunger (fed QFR on the day of testing) showed a significant preference (P < 0.01 level, denoted by **).