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Conditioned place preference or aversion as animal welfare assessment tools: Limitations in their application

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ABSTRACT

Conditioned place preference (CPP) or aversion (CPA) methods are potentially useful tools in animal welfare assessment because they permit measurement of the reinforcing properties of a stimulus in the absence of the stimulus itself. We used CPP/CPA techniques in a series of experiments to assess the preference of food restricted broiler breeders for increased food quantities or avoidance of aversive stimuli. In all experiments, 6–10 week old Ross308 pairs of broiler breeders were housed in pens divided in half. Pen sides were visually differentiated and birds were trained with different stimuli on each pen side, correcting for possible side biases, either different food amounts for CPP or aversive stimuli on one pen side and none, or 'neutral', on the other, for CPA. To test if a preference for a pen side had been formed, the pen divider was removed when no stimulus was present and the amount of time birds spent on each pen side was recorded. Each experiment had a factorial treatment structure ($n = 10$ replicate pens per treatment combination) and the proportions of time spent by birds on the 'positive' pen side (i.e. increased food amount for CPP or 'neutral' for CPA) were analysed. In experiment 1 on CPP (180 birds forming 90 pairs), three different training regimes in combination with three different testing methods were trialled: whilst during training of all birds, on one pen side the birds received the commercially recommended, restricted amount of food (R) and on the other pen side they received twice that amount (2R) (no food was present during testing). In experiment 2 (110 birds forming 40 pairs and 10 individually housed) and 3 (80 birds forming 40 pairs), further refinements were made to the experimental methods and birds were allocated to CPP treatments with food amounts 2R or 3R (vs. R) or to CPA treatments, 'social isolation' or 'unpredictable wind'. Overall, there was no evidence of aversion at testing to the pen sides with aversive stimuli during training and little evidence of preference at testing for the pen sides with increased food amounts during training. Furthermore, where statistical significance was achieved for CPP the preferences shown were very small. The most consistent result was a strong preference for the pen side birds were not previously housed on immediately before each test ($P < 0.001$ in all experiments). It appeared that birds were motivated

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to explore a location where they had not just been housed in an attempt to find food and this motivation seemed to overshadow other effects. This series of experiments demonstrates some limitations of CPP/CPA techniques for welfare assessment and the learning problems experienced by chronically food restricted animals.

1. Introduction

A major challenge in animal welfare science has been the development of reliable measures of affective state in non-human subjects. One important category of measures attempts to quantify the motivation of animals to obtain particular resources. Typically, the animal has to work, e.g. by repeatedly pressing a lever or pushing through a weighted door to obtain an immediate reward (operant or consumer demand tests, e.g. Dawkins, 1983, 1990; Mason et al., 2001; Seaman et al., 2008, reviewed by Kirkden and Pajor, 2006; Jensen and Pedersen, 2008). Animals that are prepared to work hard to obtain a resource are assumed to be motivated to gain access to that resource. They would, therefore, have improved welfare if provided with, and decreased welfare if denied, that resource. However, two similar but complementary methods are used, especially in pharmacology (e.g. Bardo and Bevins, 2000), to assess the motivation for a resource: (i) self administration (operant responding for immediate reward) which is directly analogous to the approaches described above and (ii) Conditioned Place Preference and Aversion (CPP/CPA; reviewed by Bardo and Bevins, 2000), which is different in a number of respects.

CPP/CPA is used to evaluate preferences for contextual stimuli that have been associated with a positive or negative reward, for example determining the addiction potential of drugs. In these experiments, an animal learns (through Pavlovian, or classical, conditioning) to associate a particular location (the conditioned stimulus, CS) with a particular stimulus (e.g. drug delivery, the unconditioned stimulus, US), in contrast to a location where the stimulus is absent. The location preference is then tested in the absence of the US, i.e. in extinction tests (Tzschentke, 1998, 2007). This contrasts with self-administration in which the US is presented during testing and the operant response is measured for an immediate reward. Animal welfare is more concerned with the longer term affective states of animals rather than the short term states that are often measured (Mendl et al., 2010). It is in this respect that CPP/CPA is importantly different from 'self-administration' tests of motivation: (i) Administration of certain drugs which affect the general 'mood' state of the animal by acting primarily on serotonergic pathways (such as LSD, Buspirone and Pentylenetetrazole) can elicit CPP/CPA, but not operant responding (self-administration; Bardo and Bevins, 2000). (ii) CPP/CPA is not influenced by the feedback from the 'reward' given during conventional operant tests which affect the motivation of the animal for the reward (discussed further below) thus (iii) CPP/CPA will measure the animal's overall experience of an environment or situation rather than more specific behavioural and physiological responses to a stimulus and, as a result, could be a useful tool for assessing animal welfare.

We used food quantity and lack of aversive stimuli preferences in food-restricted broiler breeder females to determine the suitability of CPP/CPA as a welfare assessment tool. Food restriction takes place in a variety of captive animals (reviewed by D'Eath et al., 2009) but is relatively most severe in broiler breeders. Broiler (meat) chickens have been intensively selected for fast growth rate and are ready to be marketed at 5–6 weeks of age. As a result, the parent stock of these broilers, called broiler breeders, also has this potential for fast growth. Broiler breeders are food restricted to reduce their growth rate and prevent problems associated with fast growth and excessive weight gain, such as lameness, premature death and poor fertility (reviewed by Mench, 2002; D'Eath et al., 2009). Restriction is at its most severe during the rearing phase when broiler breeders may only be fed 33% of what ad libitum fed birds the same age would consume (Savory et al., 1996; de Jong et al., 2002). However, restricted feeding causes a new set of problems including abnormal behaviour, such as spot pecking and polydipsia or water spillage, which suggests frustration of feeding motivation (e.g. Savory et al., 1992; Savory and Maros, 1993; Hocking et al., 1996, 2001; Sandilands et al., 2005). This has been confirmed by the finding that restricted birds will learn to peck a key at high ratios to obtain extra food (Savory et al., 1993). Taken together, this evidence indicates that food restricted birds will be chronically hungry and experiencing stress, which has a negative impact on bird welfare (Hocking et al., 1996, 2001; Savory et al., 1993, 1996; Mench, 2002).

Previous research thus provides, at first sight, convincing evidence for a negative affective state of hunger in broiler breeders (e.g. Dawkins, 1990). However, as mentioned above, measuring hunger through self administration by presenting extra food, for example in a preference or operant conditioning task (Savory et al., 1993; Savory and Lariviere, 2000) or a rate of eating task (Sandilands et al., 2005, 2006), affects the animal's context. This changes the situation from an animal that has to cope because it has no access to food to an animal that is aware that additional food is available (even if they must work to get it). In addition, sensory cues from the food, such as sight and smell, may increase the motivation to feed, while without the cues, this motivation may remain low (i.e. 'out of sight is out of mind', Warburton and Mason, 2003). Thus, (i) the potential to obtain additional food is in itself likely to increase feeding motivation and (ii) once the first food has been consumed motivation may be increased as a result of positive feedback (Day et al., 1997), until at least a state of satiety is reached. The risk is, therefore, that broiler breeders will make impulsive choices on the basis of an immediate payoff and measurements will reflect short term rather than long term affective states. In the past, a number of physiological measures of hunger have been proposed (e.g. as discussed by de Jong et al., 2002).

These have been criticised on the grounds that they may actually relate to changes in energy substrate concentrations in the body, which vary during the day depending on meal pattern and on weight, age and lipid reserves. These measures may, therefore, not reflect the animals' experience of hunger at all (for review see D'Eath et al., 2009). Thus, behavioural measures of the affective state associated with food deprivation that do not involve presenting extra food are needed.

Our objectives in this series of experiments were, (i) to develop a methodology for consistently achieving CPP in broiler breeders, (ii) to test the hypothesis that broiler breeders would demonstrate a CPP for a location associated with increased, compared to recommended restricted, food rations and (iii) to test the hypothesis that broiler breeders would form a CPA to a location associated with an aversive stimulus with the intention that CPA could be used to calibrate the strength of the CPP associated with additional food.

2. Materials, animals and methods

2.1. General methods

All birds were non-beak trimmed Ross308 broiler breeder hens and were obtained at one day of age. They were housed in floor pens (1 m × 2 m) covered in wood shavings until 4 (8 for experiment 2) weeks of age. The lighting schedule for the first day was 23.5L:0.5D hours light:dark, which was then gradually reduced to 8L:16D over 10 days. Temperature followed commercial recommendations, decreasing from around 30 °C at bird level at one day old to around 20 °C by four weeks of age. Chicks were given ad libitum water from bell drinkers and were fed chick starter crumbs for the first three weeks, chick starter pellets for the following three weeks and then grower pellets (all ABN, Cupar Mills, Fife) from the beginning of six weeks of age to the end of the trial. Food was provided ad libitum for the first 7 days and then in restricted amounts given at 09:00 h each day that were gradually increased from 26 to 44 g per bird per day by the beginning of the 5th week, as per the Ross308 parent stock guidelines (Aviagen, 2007).

2.2. Ethical considerations

Birds never had restriction placed on their water intake and were housed on a bedding of wood shavings. Food restriction is likely to result in hunger, but research into animal welfare problems often faces the difficulty that the problem must be recreated in the laboratory in order to study it. At any one time there are an estimated 7.5 million broiler breeder chickens in the UK alone (Defra, 2006; Sandilands et al., 2006) all of which are restricted-fed (R) during rearing. The levels of food restriction we imposed were less severe than that used routinely in the poultry industry, with birds receiving at least the industry recommended level (R) of food or some multiple of this, e.g. 2R indicates double the commercial ration for a bird of that age (CPP: alternation of R and 2R or 3R, CPA: 1.5R or 2R). All procedures in this experiment were carried out under Home Office Licence and with the SRUC Animal Experiment Committee's approval and birds were checked a minimum of three times per day.

3. Experiment 1

3.1. Animals, materials and methods

3.1.1. Overall experimental design

A 3 × 3 factorial randomised block experiment was conducted using three different training regimes, switching between the two pen sides (i.e. the two different stimuli) every 2, 4 or 6 days. These were used in combination with three different testing times, with an observation period of 10 min starting 2, 6, or 24 h after birds were last fed, to find the best methodology for establishing CPP for double food rations compared to the recommended amount (2R vs. R; Table 1). Ninety pens were used divided into 10 blocks to control for location within the shed. Each block contained nine pens, one for each treatment.

3.1.2. Animals and housing

Non-beak trimmed Ross308 broiler breeder females (180) were received from Vion (Moy Park, Ireland) as day old chicks. At 4 weeks of age, all birds were weighed, wing tagged (1 cm × 1 cm padlock-style tags, Roxan Developments Ltd., UK) and paired according to matched body weight. Ninety floor pens (1.5 m × 1.0 m) housed one pair of birds each. One bird of each pair was randomly selected to be the 'marked' bird (marked with livestock spray before testing), while the other was unmarked during testing. Each pen was bedded with wood shavings and provided water through a nipple drinker. Food was presented in four small (9 cm width × 7.5 cm height × 5.5 cm depth) semi-circular food cups, two on the left side of the pen wall and two on the right. Starting at 6 weeks of age, birds began to receive either R or 2R, depending on their treatment schedule (see below). All birds were weighed every two weeks for the duration of the trial (16 weeks). Two birds from two different pairings had to be culled while the experiment was already advanced and were not replaced because replacement birds would have missed training time. The remaining bird of each pair was singly housed for the duration of the study but had visual contact with other birds at all times.

3.1.3. Testing to get pre-training baseline measures of initial side preferences

At five weeks of age, bird pairs were removed from the pen, one bird was marked with livestock spray and both were placed at the front of their own pen in a start box (40 cm × 40 cm × 40 cm) that had a clear sliding door allowing visual access to the pen. The pen door was opened and replaced by the start box which covered the entire opening. After being allowed to settle for 2 min, the clear sliding door of the start box was opened and the birds could leave the box. Testing lasted 10 min from the time the start box door was opened. The duration of time spent on each side was recorded for both birds to estimate baseline side preferences. Birds spending more than 60% of their time on one side of the pen were considered to have a pre-existing side bias.

Table 1
An outline of conditions used on the two pen sides and other factors varied in each of the three experiments examining conditioned place preference (CPP). Commas are used to distinguish between different levels for the other factors tested in the experiments whilst "vs." is used to indicate the different conditions on the two pen sides during training.

Expt.	Visual cues on pen sides	CPP			
		Food amounts on pen sides	Training regime (days)	Time to testing after feeding (h)	Number of tests (days between tests)
1	Horizontal stripes vs. vertical stripes	2R vs. R	2, 4, 6	2, 6, 24	6 (12)
2	Horizontal stripes vs. vertical stripes	2R vs. R, 3R vs. R	1, 2	24	4 (8)
3	Horizontal strips vs. vertical stripes, black solid vs. white solid	3R vs. R	2	6	3 (12)

R = commercially recommended and restricted food amount; training regime = days between pen side switches.

Pairs of birds in the same pen tended to behave similarly and to have the same pre-existing side bias. However, when one bird showed a side bias (as defined above) and the other did not, the pen, or pair, was still considered to have a side bias. On this basis, thirty-five pairs of birds showed a right bias, 29 pairs showed a left bias and 26 pairs showed no bias. Treatments were then allocated to pairs balanced as much as possible for estimated pre-existing side biases.

3.1.4. CPP treatment

When birds were 6 weeks of age the pens were divided into two with a wire mesh frame and plywood panels were attached to the back and one wall of each side of each pen. Panels had black and white stripes, vertical for one and horizontal for the other side of each pen. During training, a pair of birds would always receive food allowance R on the pen side with either the vertical or horizontal stripes and then receive 2R on the other pen side (Fig. 1a). To enable the 90 pairs of birds to be tested within the time available, birds were allocated to three scheduling groups, balanced for between bird treatment combinations and block, which were tested on subsequent days. Allocations of horizontal and vertical stripe panels and of R and 2R to left and right pen sides were balanced with each other and across other treatments and (as far as possible) across estimated pair side biases.

3.1.5. Training regime and testing

Pairs of birds were allocated to one of three training regimes in which they alternated between pen sides with the different food treatments (either R or 2R) every 2, 4 or 6 days (n = 30 pairs each) for 72 days. Birds were moved from one pen side to the other in the afternoon. Order of food stimuli presented first, R or 2R, was balanced across all treatments in the study and, as far as possible, estimated side biases. During training, all pairs of birds were tested every 12th day for six tests in total in order to examine the effect of test number on learning. The experiment ended after the 6th test when the birds were approximately 17 weeks of age.

Pairs of birds were allocated to one of three test times of day, starting either 2, 6 or 24 h since last being fed. Pairs of birds from a scheduling group were all tested on the same day, with the '24' h birds tested at normal feeding time (i.e. 09:00 h) and then fed immediately after testing was complete and the other pairs of birds tested 2 or 6 h after normal feeding time.

At each test, birds were removed from the pen, placed in the start box and tested as described above for baseline side preference measurement. Livestock marker was re-sprayed on the marked bird if needed. The pen divider was removed and food cups were emptied of any debris (no food ever remained in the cups) and replaced while birds were in the start box and then left there for the duration of the test (i.e. 10 min). After the test, the pen divider was replaced and the birds were returned to the appropriate pen side according to their treatment schedule.

During testing the pen was visually divided down the middle into right and left sides by marked points on the pen frame as reference points for the division. The pen side the marked and unmarked birds were situated on was determined by the position of both feet – whether both feet were on the right side of the middle divide or whether both feet were to the left side of the middle divide. A bird was not recorded as changing pen sides until both feet crossed the middle divide. Birds were continuously recorded during the test in order to measure the duration of time spent on each side. After testing, in accordance with their pen switching schedule, pairs of birds on the 2 h and 6 h test time of day were placed in the new pen side to begin the next training series. Pairs of birds on the 24 h test time of day received their ration on the old pen side before being moved to the new pen side later in the day.

3.1.6. Statistical analysis

To examine effects on birds' side preferences two different measurements were analysed. (1) *Comparison with pre-training baseline* for which the proportion of time each bird spent on the 2R (or 'positive') pen side during their baseline measurement was subtracted from the proportion of time the birds spent on the positive pen side at each test after training. Such a difference in time spent on the positive pen side after training compared to baseline is a measure commonly used in pharmacological studies that apply CPP/CPA techniques (e.g. Jarosz et al., 2006). (2) *Comparison with chance expectation* (i.e. *0.5*) for which 0.5 was subtracted from the proportion of time birds spent in the positive pen side at each test after training. This measure is a comparison of the time spent on that pen side with the expected time assuming that birds are indifferent to pen sides (i.e. that they move at random and have no underlying side bias). The justification for using this

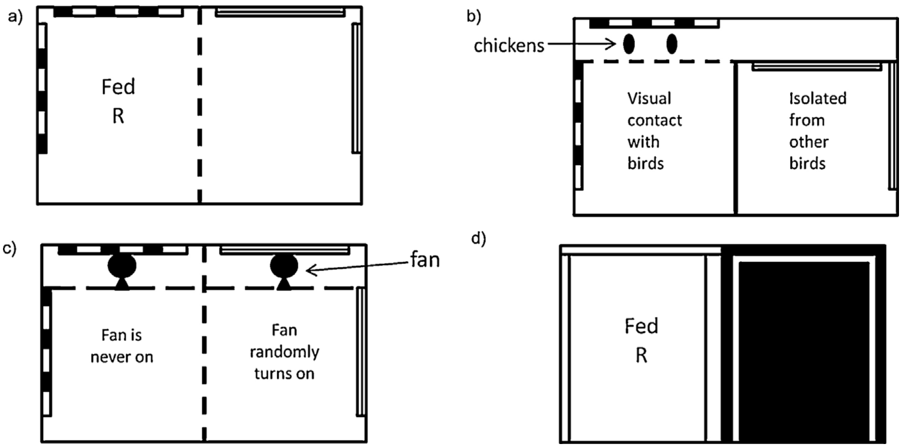

Fig. 1. Pen set up for (a) original visual cues (all experiments but 2R used in Experiments 1 and 2 and 3R in Experiments 2 and 3), aversion cues, (b) social isolation (Experiment 2), (c) unpredictable wind (Experiments 2 and 3), and (d) enhanced visual cues (Experiment 3).

second measure is twofold. Although a measure that adjusts for true underlying side biases for birds at the time of each test is likely to be superior, in practice this is carried out by using an initial estimate of side bias for each bird which may be an inaccurate representation of the truth as it will inevitably be measured with error, and, furthermore, there is no guarantee that true underlying side biases remain constant throughout subsequent testing.

Both measures were analysed by fitting Linear Mixed Models (LMMs) using Residual Maximum Likelihood (REML) in Genstat (14th edition 2011) as residuals from fitted models suggested that alternative methods such as transforming the data were not required. Fixed effects included in the models were test number (1...6), training regime (2, 4, 6), time of day of test (2, 4, 24) and pen side (2R or R) the birds were housed on immediately before each test, including all 2 way interactions between these factors. Random effects included block (1...10), scheduling group (1...3), pen (1...90) (i.e. bird pair), and bird and interactions of these with test number (1...6). Analyses focused on differences between the levels of the different fixed effects, and, also on whether pertinent model estimates were greater than 0, as this indicates a preference for the 'positive' (2R) side of the pen. The departure between a model estimate and 0 was tested by omitting the overall constant from the model and testing a covariate in the model before all other fixed effects which took the value 1 when the data coincided with the factor level being tested, or 0, otherwise. Statistical tests, with significance at the 5% level, were based on approximate *F* tests when these were available comparing *F* statistics to the *F* distribution, but otherwise Wald tests were used in which the Wald statistic was compared to the χ^2-distribution. In approximate *F* tests, denominator degrees of freedom are estimated and may not be whole numbers when fitted factors are not perfectly balanced/and or when effects are estimated from more than one level of the random hierarchy in the model. Estimated means and standard errors (SEs) from the LMMs are reported for the 9 treatments corresponding to the interaction between training regime and time of day of test (averaged over the other fixed effects) and for other significant main effects.

3.2. Results

For comparison with pre-training baselines, there were no significant effects on the proportion of time spent on the 'positive' (2R) pen side of training regime, time of day tested or any interactions between them (Fig. 2a), nor was there any effect of test number or interaction of these with test number. Furthermore, although all these birds were

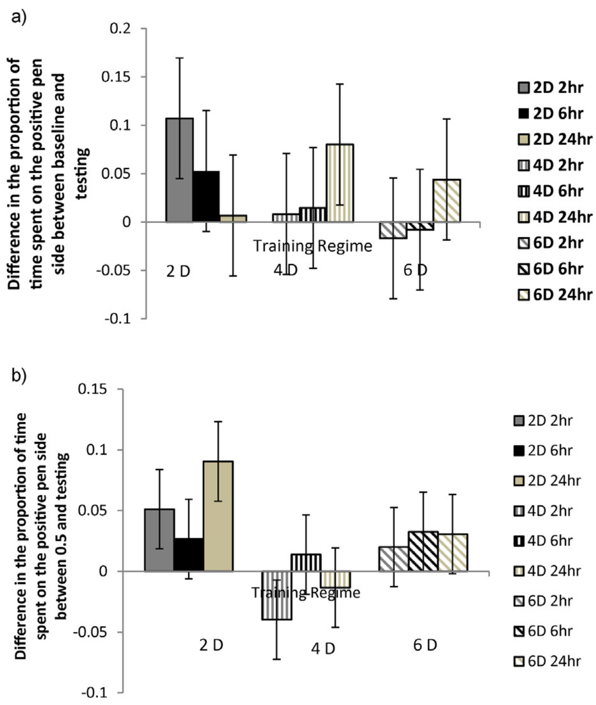

Fig. 2. The differences (predicted means ± SE) between the proportions of time spent on the positive pen side and (a) baseline measures and (b) 0.5 or chance for treatments in Experiment 1.

being offered an increased food amount during training, there was no evidence that, over all the treatments and tests, they were spending more time during testing on the 'positive' (2R) pen side than on the other (R) side compared to their pre-training baseline measurements ($F_{1,\ 8.8}=2.52$, $P=0.148$). The largest effect was of the pen side that the birds had been housed on immediately before each test with birds spending less time on this side than on the other (more novel) side ($F_{1,\ 229.74}=229.74$, $P<0.001$). The proportion of time spent on the 'positive' pen side (2R) decreased (-0.127 ± 0.027) or increased (by 0.191 ± 0.027) compared to the baseline, depending on whether birds were housed on that side immediately before each test or not.

For comparison with chance expectation (0.5), there was a marginally insignificant effect on the proportion of time spent on the 'positive' (2R) pen side of training regime ($F_{2,\ 76.1}=3.12$, $P=0.050$; Fig. 2b), with birds switching between R and 2R every 2 days spending more time on the positive pen side than birds switching every 4 days, and birds switching every 6 days being intermediate (2: 0.056 ± 0.020, 4: -0.013 ± 0.020, 6: 0.028 ± 0.020). However, even the preference shown for the 2R pen side for birds trained with 2 day switching was very small. As above, there was no evidence that, over all the treatments and tests, birds were spending more time during testing on the 'positive' (2R) pen side than on the other (R) side compared to 0.5 ($F_{1,\ 2.0}=4.54$, $P=0.167$). There were no significant effects of time of day tested or test number or interaction of these with training regime. Again, the largest effect was of the pen side that the birds had been housed on immediately before each test ($F_{1,\ 342.1}=353.27$, $P<0.001$). The proportion of time spent on the 'positive' pen side (2R) decreased (by -0.139 ± 0.016) or increased (by 0.186 ± 0.016) compared to 0.5, depending on whether birds were housed on that side immediately before each test or not.

3.3. Discussion

This experiment was designed to determine the best training and test methodology for establishing a CPP to a location associated with an increased food ration (2R) in food restricted birds. However, none of the treatments showed a strong preference: the increased proportion of time spent on the 2R side was <0.11 over all 9 treatments and both measurements and the differences between treatments were very small. The most significant result was a preference for the 'less familiar' pen side, i.e. the side birds had not been housed on just before each test. After the birds consumed their daily food ration (within 20 min), they spent a large part of the day foraging unsuccessfully and this likely results in high motivation to forage elsewhere. This effect may have overshadowed any potential positive affective states associated with the pen side where 2R was provided.

In traditional CPP studies, there is usually an all-or-nothing scenario where the animal gets the positive stimulus in one location and nothing in the other. In contrast, our birds received feed in both locations; both quantities were considerably less than ad libitum intake and were consumed within 20 min. It is possible that severely food restricted birds have such a high feeding motivation that getting any food is positive. As a result, they might not consider an environment with 2R (much) more rewarding than an environment with R and this may have prevented the expression of CPP. A larger food portion, such as 3R, might then give better results when contrasted with R. For the second experiment therefore, a larger contrast (3R vs. R) was also investigated. A 2 day training regime was selected because this was the most successful in the first experiment, and a 1 day training regime was added as switching pen sides every day might decrease the 'less familiar' pen side effect observed in the first experiment. Birds were tested 24 h after last being fed because, although not statistically significant, this (when averaged over other factors) was the most successful test time of day in the first experiment. As birds had not appeared to be learning as the tests progressed in Experiment 1, we decreased the time intervals between tests to every 8 days and carried out just 4 tests. Birds were also tested at an older age (10 weeks) because feed restriction is more severe for older birds, which might increase the motivation for a larger food ration.

We had initially planned in future experiments to titrate between an environment with an aversive stimulus but increased food rations compared to an environment with the restricted ration but no aversion in order to determine a relative measure of hunger. However, there are indications that learning a task involving food is especially difficult for severely feed restricted birds (Buckley et al., 2011). We introduced, therefore, in the second experiment, aversive stimuli unrelated to food to investigate whether food restricted birds could form a negative association (CPA).

4. Experiment 2

4.1. Animals, materials and methods

4.1.1. Overall experimental design

A 2×4 incomplete factorial randomised block experiment was conducted to measure CPP/CPA of birds for the 'positive' (increased food amount/non-aversive or 'neutral') pen side. Two training regimes (switching pen sides every 1 or 2 days) with two CPP feed treatments (2R vs. R or 3R vs. R) were used. In addition, we trialled two CPA treatments to determine if food restricted birds (fed at 1.5R, switching pen sides every 2 days) could form an aversion to a location associated with either social isolation or wind blowing for 15 min periods per hour on a random schedule (Table 2). 60 pens were used divided into 10 blocks (2 per room), to control for location in the shed. Each block contained six pens, one for each treatment.

4.1.2. Animals and housing

Non-beak trimmed Ross308 broiler breeder females (110) were received from Aviagen (Stratford, UK) as day old chicks. They were housed as described above until 8 weeks of age when all birds were handled and allocated in pairs to sixty floor pens all as described in experiment 1 except that for the social isolation CPA treatment 10 birds were housed individually. Pens were bedded and birds were marked, watered and fed similarly to Experiment 1. Starting at 10 weeks of age, birds began to receive either (for CPA) 1.5

Table 2
An outline of conditions used on the two pen sides and other factors varied in each of the three experiments examining conditioned place aversion (CPA). Commas are used to distinguish between different levels for the other factors tested in the experiments whilst "vs." is used to indicate the different conditions on the two pen sides during training.

Expt.	Visual cues on pen sides	CPA				
		Aversion on pen sides	Food amount on pen sides	Training regime (days)	Time to testing after feeding (h)	Number of tests (days between tests)
1	Horizontal stripes vs. vertical stripes	n/a	n/a	n/a	n/a	n/a
2	Horizontal stripes vs. vertical stripes	Social isolation vs. see other birds, Unpredictable wind vs. none	1.5R	2	24	4 (8)
3	Horizontal strips vs. vertical stripes, black solid vs. white solid	Unpredictable wind vs. none	2R	2	6	3 (12)

R = commercially recommended and restricted food amount; training regime = days between pen side switches.

times the recommended ration (R) or (for CPP) R, 2R or 3R, depending on their treatment schedule (see below). All birds were weighed every two weeks for the duration of the trial (5 months). During the experiment, one bird had to be culled and was not replaced; the remaining bird of the pair was kept singly housed for the duration of the study but had visual contact with other birds at all times.

4.1.3. Testing to get pre-training baseline measures of initial side preferences

Testing for baseline measures of initial side preferences was conducted at 9 weeks of age, but otherwise in the same way as in Experiment 1 and 24 pairs/pens had a left bias, 32 had a right bias and 4 had no bias. Treatments were allocated to pens balanced as much as possible for estimated pre-existing side biases.

4.1.4. CPP/CPA treatments

When birds were 10 weeks of age, the pens were divided into half and visually differentiated in the same way as in Experiment 1. For CPP treatments, a pair of birds would receive R on one pen side and 2R or 3R on the other side during training (Fig. 1a). Birds on the CPA treatments received an aversive stimulus on one pen side while the other side remained neutral during training (Fig. 1b and c).

For the CPA part of the experiment, the first aversive stimulus was social isolation, applied to 10 single birds. During training, on one side of the pen the test bird had visual access to 2 companion birds who were housed behind a wire mesh screen whilst on the aversive side of the pen all visual access to other birds was blocked by sheets of black tarpaulin attached to the pen sides (Fig. 1b). The second aversive stimulus was unpredictable wind, applied to 10 pairs of birds. Fans were placed in the back of both pen sides behind wire mesh screens. However, only the fan in the aversive pen side turned on randomly for 15 min every hour (Fig. 1c).

4.1.5. Training regime and testing

We used two training regimes with the CPP treatments with 40 pairs, i.e. alternating between R and 2R or 3R either every day or every two days (n = 10 pairs per treatment). Birds on the CPA treatments alternated between the aversive and neutral pen sides every two days. The order of CPP/CPA stimuli presented first was balanced across all other factors in the study and, as far as possible, estimated side biases. Birds were tested every 8 days for 32 days (4 tests in total) and the experiment ended after the 4th test when the birds were approximately 16 weeks of age.

Birds in Experiment 2 were all tested at 24 h since last being fed. Testing for the CPP study was conducted in the same way as in Experiment 1. As usual in CPA studies, birds were tested in absence of the 'wind' aversion, i.e. fans were turned off during testing. For 'social isolation', to avoid interference of companion birds, these were removed during testing, similar to CPP studies (removal of the stimulus). The same measurements as in Experiment 1 were collected for both the CPP and CPA tests in Experiment 2.

4.1.6. Statistical analysis

The same methods were applied as described for experiment 1, analysing the difference between the proportion of time spent on the 'positive' (2R or 3R for CPP, non aversive or neutral for CPA) pen side and baseline estimates or 0.5. Fixed effects included in the models were test number (1...4), training regime (1, 2), CPP/CPA treatment which had 4 levels (2R vs. R, 3R vs. R, social isolation vs. visual access to other birds, unpredictable wind vs. none) and pen side (positive or negative) the birds were housed on immediately before each test, including all 2 way interactions between these factors. Since the training regime and the CPP/CPA treatment were confounded (the 1 day training regime was only trialled for the two CPP treatments) the factors were tested in both orders in order to investigate each factor adjusting and not adjusting for the other. Random effects included room (1...5), block (1...10), scheduling group (1...3), pen (1...60), and bird and interactions of these with test number (1...4).

4.2. Results

For comparison with pre-training baselines, there were no significant effects on the proportion of time spent on the 'positive' pen side of CPP/A treatments, training regime

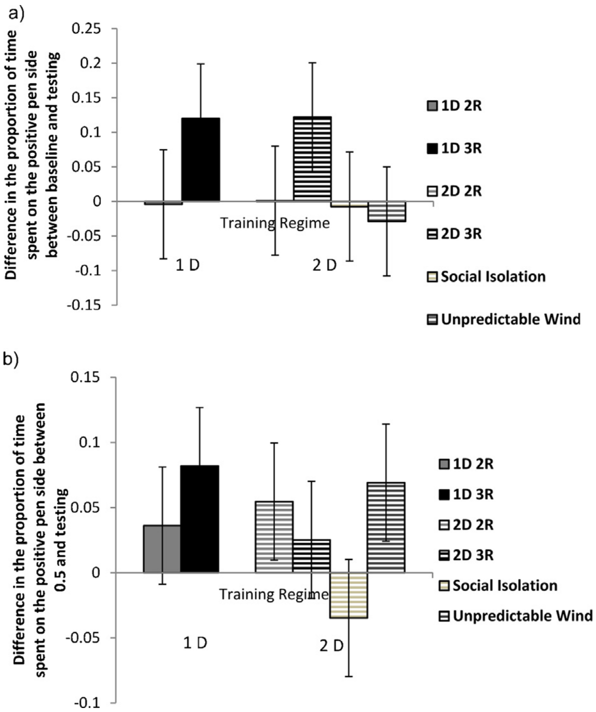

Fig. 3. The differences (predicted means ± SE) between the proportions of time spent on the positive pen side and (a) baseline measures and (b) 0.5 or chance for treatments in Experiment 2.

or any interactions between them (Fig. 3a). There was also no significant effect of test number but the interaction between test number and training regime was marginally significant ($F_{3,\,147.4} = 2.79$, $P = 0.043$), but this was not indicative of a clear 1 day or 2 day trend favouring either training regime. There was no evidence that, over all the treatments and tests, birds were spending more time during testing on the 'positive' pen side than on the other side compared to their pre-training baseline measurements ($F_{1,\,3} = 0.61$, $P = 0.491$). As in Experiment 1, the largest effect was of the pen side that the birds had been housed on immediately before each test with birds spending less time on this side than on the other (more novel) side ($F_{1,\,168.5} = 62.15$, $P < 0.001$). The proportion of time spent on the 'positive' pen side decreased (by -0.065 ± 0.060) or increased (by 0.104 ± 0.060), depending on whether birds were housed on that side immediately before each test or not.

For comparison with chance expectation (0.5), there were no significant effects on the proportion of time spent on the 'positive' pen side of CPP/A treatments, training regime or any interactions between them (Fig. 3b). As above, there was also no significant effect of test number but the interaction between test number and training regime was marginally significant ($F_{3,\,147.1} = 2.83$, $P = 0.041$) but again this was not indicative of a clear 1 day or 2 day trend favouring either training regime. There was no evidence that, over all the treatments and tests, birds were spending more time during testing on the 'positive' pen side than on the other side compared to 0.5 ($F_{1,\,4} = 2.40$, $P = 0.196$). Again, the largest effect was the pen side the birds had been housed on immediately before each test ($F_{1,\,186.8} = 69.96$, $P < 0.001$). The proportion of time spent on the positive pen side decreased (by -0.063 ± 0.036) or increased (by 0.114 ± 0.036), depending on whether birds were housed on that side immediately before each test or not.

4.3. Discussion

Three times the commercially restricted food amount (3R) is almost equivalent to ad libitum feeding (Buckley et al., 2011). However, this considerable increase (from 2R to 3R) in the contrast between food allowances did not have the expected effect on the observed CPP. The failure of birds to learn the CPP tasks is, therefore, unlikely to be a result of inadequate differences between food treatments. In addition, with CPA, birds did not learn to avoid a location associated with an aversive stimulus when both pen sides supplied the same amount of feed. Although the aversion in our experiment was either present or not, similar to many 'all or nothing' situations in other CPP/CPA studies (e.g. Bardo and Bevins, 2000), we failed to establish a consistent CPA in our birds. As with Experiment 1, the most significant result was a preference for the 'less familiar' pen side, i.e. the side birds had not been housed on just before each test, and birds appeared motivated to explore that area, possibly in an effort to find more food as discussed above.

Observations of bird behaviour showed that they spent most of their time foraging, i.e. their attention was directed to the floor. However, the floors on the different pen sides were not visually distinct in our experiments and it is possible that the visual differences on the walls alone were inadequate cues for the birds to learn the distinction. A third experiment was, therefore, designed to test the effect of increased visual contrast between pen sides on the establishment of CPP/CPA. In this experiment CPP/CPA treatments chosen were 3R vs. R and unpredictable wind because, although not statistically significant, these were the most promising in experiment 2. However, for unpredictable wind the severity of the treatment was increased by switching the fan on more frequently. As training regime had little effect in experiment 2 all birds were trained switching pen sides every 2 days, and as testing the birds every 8 days did not improve preferences for the 'positive' pens side, all birds were tested every 12 days (as in Experiment 1) to allow for more training with each pen side before a test (3 exposures to each pens side opposed to 2) for 3 tests in all. Time of testing was changed to 6 h after feeding as opposed to 24 h after feeding (or testing immediately before the morning meal) because it was observed that the birds became quite frantic when they were not immediately fed in the morning and there was concern that not only were the birds not focused on the test but that they may injure themselves with this behaviour. Birds began training at 6 weeks of age because younger birds (as observed in Experiment 1) seemed less unsettled during testing than older birds (as observed in Experiment 2). Finally, in order to increase the accuracy of estimated baselines, the baseline measurements were made twice for each bird, instead of once as in the first two experiments.

5. Experiment 3

5.1. Animals, materials and methods

5.1.1. Overall experimental design

A 2 × 2 factorial randomised block experiment was conducted to test the effects of increased visual distinction on the establishment of CPP/CPA in broiler breeders. One of the contrasts between visual cues of the two pen sides was identical to that used in Experiments 1 and 2 while the other contrast was made stronger by making the floor and pen walls either white or black (which were balanced between positive and negative treatments). The CPP treatment was 3R vs. R and for the CPA treatment, to increase wind aversion, fans were switched on randomly during around 50% of daylight hours (in contrast to 25% in Experiment 2). All birds were trained and tested the same way, switching pen sides every 2 days and testing 6 h after feeding. This resulted in 4 treatments, 2 CPP treatments, i.e. 3R vs. R with the either original or with enhanced visual environmental cues, and 2 CPA treatments, i.e. unpredictable wind vs. no wind with either the original or with enhanced visual environmental cues (Tables 1 and 2). 40 pens were used divided into 10 blocks, to control for location in the shed. Each block contained 4 pens, one for each treatment.

5.1.2. Animals and housing

Non-beak trimmed Ross308 broiler breeder females (80) were received from Aviagen (Stratford, UK) as day old chicks. They were housed as described above until 4 weeks of age when all birds were handled and allocated in pairs to forty floor pens (2.0 m × 1.0 m) all as described in Experiment 1. Pens were bedded and birds were marked, watered and fed similarly to Experiments 1 and 2. Starting at 6 weeks of age, birds began to be fed alternating between R and 3R every 2 days (CPP), or they were fed 2R (CPA). All birds were weighed every two weeks for the duration of the trial which was 3 months. No birds were culled during the experiment.

5.1.3. Testing to get pre-training baseline measures of initial side preferences

Testing for baseline measures of initial side preferences was conducted in the same way as in Experiments 1 and 2 except that each pair was measured twice in order to increase accuracy of the estimated baseline measures for each bird. Overall 16 pairs were considered to have a left bias, 13 had a right bias and 11 had no bias. Treatments were then allocated to pairs balanced as much as possible for estimated pre-existing side biases.

5.1.4. CPP/CPA treatments – original and enhanced environmental cues

When the birds were 6 weeks of age, the pens were divided into two and plywood panels were attached to the backs and sides of the pens to differentiate the environments. The environmental cues used in half of the pens were identical to those used in Experiments 1 and 2. To test the effects of enhanced visual cues, the other pens were divided into two by a solid divider in which the front 1/3 could be removed for testing; one side of the solid divider was painted white, the other side was black. Additionally, solid black or white panels were attached to the back and sides of the pens and solid black or white vinyl flooring was put on the floor and covered with a thin layer of wood shavings (Fig. 1d).

5.1.5. Training regime and testing

During CPP training, a pair of birds received food allowance R on the pen side with either the vertical or horizontal stripes or with either the solid black or the solid white panels for 2 days, then 3R on the other pen side for 2 days. During CPA training birds were exposed to the aversive wind stimulus on one pen side but not on the other. The order of CPP/CPA stimuli presented first was balanced across all other factors in the study and, as far as possible, estimated side biases. All birds were trained for 12 days then tested and this was repeated twice. The experiment ended after the third test when birds were approximately 12 weeks of age.

Birds in Experiment 3 were all tested at 6 h since last feed. Testing for the CPP study was conducted in the same way as in Experiments 1 and 2. The birds in the enhanced environmental cue pens had the front third of the solid pen divider removed before beginning the test. The same measurements were collected as in Experiments 1 and 2.

5.1.6. Statistical analysis

The same methods were applied as described for experiments 1 and 2, analysing the difference between the proportion of time spent on the 'positive' (3R for CPP, non-wind or neutral for CPA) pen side and baseline estimates (average of 2) or 0.5. Fixed effects included in the models were test number (1...3), CPP/CPA treatment (3R vs. R, unpredictable wind vs. none), visual cue pattern (horizontal stripes vs. vertical stripes, black solid vs. white solid) and pen side (positive or negative) the birds were housed on immediately before each test, including all 2 way interactions between these factors. Random effects included block (1...10), scheduling group (1...3), pen (1...40), and bird and interactions of these with test number (1...3).

5.2. Results

For comparison with pre-training baselines, there were no significant effects on the proportion of time spent on the 'positive' pen side of the different visual cue patterns. There was a marginally significant ($F_{1,\,33.7} = 4.59$, $P = 0.040$) effect of CPP/A treatments, with birds on the CPP (3R vs. R) treatment spending more time on the positive pen side (0.121 ± 0.066) than birds on the CPA (wind vs. none) treatment (−0.054 ± 0.066) (Fig. 3a). There was evidence that birds on the CPP treatment were spending more time during testing on the 'positive' pen side than on the other side compared to their pre-training baseline measurements ($\chi^2_1 = 7.67$, $P = 0.006$). There was no significant effect of test number or interactions with test number. Again, birds spent less time on the pen side they were most recently housed on before each test than on the less familiar side ($F_{1,\,101.1} = 75.96$, $P < 0.001$). The proportion of time spent on the positive pen side decreased (by −0.208 ± 0.058) or increased (by 0.275 ± 0.058), depending on whether birds

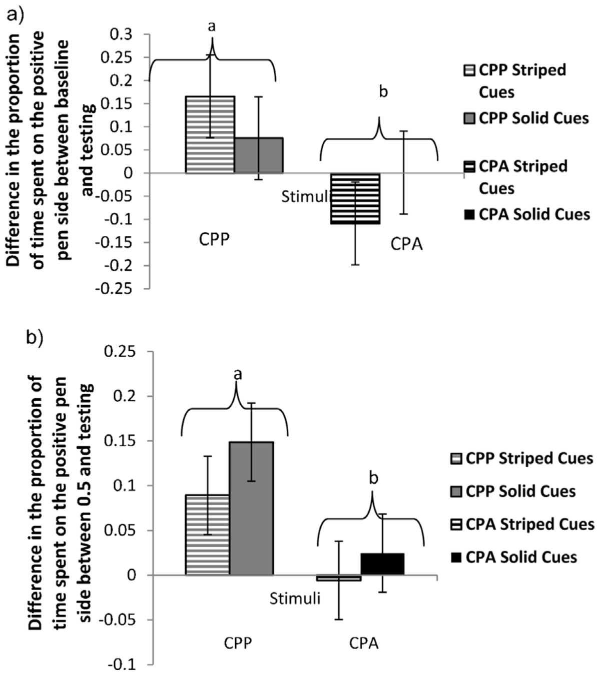

Fig. 4. The differences (predicted means ± SE) between the proportions of time spent on the positive pen side and (a) baseline measures and (b) 0.5 or chance for treatments in Experiment 3. Different letters denote significant differences between CPP and CPA (a) $P = 0.040$ and (b) $P < 0.001$.

were housed on that side immediately before each test or not Fig. 4.

For comparison with chance expectation (0.5), there were no significant effects on the proportion of time spent on the 'positive' pen side of the different visual cue patterns. There was a highly significant ($F_{1, 90.9} = 13.80$, $P < 0.001$) effect of CPP/A treatments, with birds on the CPP (3R vs. R) treatment spending more time on the positive pen side (0.119 ± 0.037) than birds on the CPA (wind vs. none) treatment (0.009 ± 0.037) (Fig. 3b). There was evidence that birds on the CPP treatment were spending more time during testing on the 'positive' pen side than on the other side compared to 0.5 ($\chi^2_1 = 35.96$, $P < 0.001$). There was no significant effect of test number or interactions with test number. Again, birds spent less time on the pen side they were most recently housed on before each test than on the less familiar side ($F_{1, 100.2} = 277.89$, $P < 0.001$). The proportion of time spent on the positive pen side decreased (by -0.204 ± 0.037) or increased (by 0.333 ± 0.037), depending on whether birds were housed on that side immediately before each test or not.

5.3. Discussion

Birds on the CPP treatment appeared to do better at the task (spent more time on the positive/neutral pen side) than birds on the CPA treatment. This indicates that birds learnt the association between an increased amount of food and a location more easily than an aversive stimulus and a location. This is opposite to what we had conjectured in the previous experiments. However, food is a very important resource for these birds and they are highly focused on foraging to find more food which may have led to a greater attendance to the food provided than to the potentially aversive stimuli. Still, the overall increase in proportion of time spent on the positive pen side compared to the 'non-positive' side was not large (0.12 for CPP), indicating that our enhancements to the environment did not drastically improve the birds' preferences.

Enhancing the visual cues did not result in a statistically significant increase in preference for the positive pen side although the preference was on average larger for the solid black and white cues than for the horizontal and vertical stripe cues. As the contrast between pen sides in both cue treatments was quite large, it seems highly unlikely that the birds would be unable to distinguish between the 2 environments during training and testing. Finally, it was the pen side the birds had not been housed on immediately before the test that again had the largest effect. This again seems to emphasise the motivation of the birds to search for food, mainly in a location where they had not foraged recently in order to maximise their chances of finding food and reducing hunger.

6. General discussion

We decided to analyse the data in two different ways. One was comparing the proportion of time spent on the 'positive' pen side at each test to an estimated pre-training baseline to determine if changes in behaviour occurred after training compared to previous behaviour, a method commonly used in pharmacology studies (e.g. Bardo and Bevins, 2000; Sakoori and Murphy, 2008; Vindenes et al., 2009; Zakharova et al., 2009). The other was comparing the proportion of time spent on the 'positive' pen side to 0.5 to determine if a change in behaviour had occurred compared to that which would be expected by chance. The results generated from these two methods were in agreement in some analyses but there were discrepancies in other analyses. In all 3 experiments, comparison of proportions to pre-training baselines resulted in substantially more variation than comparisons with 0.5 and so the latter approach gave more sensitive analyses. This was so even in the third experiment where measurements of the baselines had been repeated in order to increase their accuracy. This indicates that in some cases, such as the experiments described here, comparisons with 0.5 may be a better way to analyse data and should be considered as a method of analysis for future CPP/CPA studies.

In all experiments, the variable with by far the largest effect on birds' pen side preference was the side they had been housed on immediately previous to testing. This was so at all ages birds were trained and tested, at all different levels of contrast between food availability or duration of fans blowing, at both levels of visual cues and after all the various training regimes and at all hours after food supply that the birds were tested. These findings have important implications for conducting experiments such as those reported here. Specifically, experimental designs need to take this effect into account balancing for it in such a way that it is not confounded with experimental treatments. The urge of hungry animals to explore novel areas for food is, of course, the likely explanation for this finding ('optimal' foraging strategy, e.g. Stephens & Krebs, 1986; Andersson et al., 2001). Chronic stress does not appear to prevent motivation to explore novel environments. For example, the chronically stressed rats in Wright and Conrad (2005)'s study were still motivated to explore novel environments, even though their spatial memory was inhibited (see also discussion below). This mechanism may well have largely overshadowed any positive or negative affective states associated with pen sides providing different stimuli.

Unless this effect was taken into account in the statistical analyses, none of the other treatment factors (e.g. different training regimes or contrasting food availability) significantly affected pen side preference. When this effect was taken into account, treatment effects on pen side preference were observed that were sometimes statistically significant. However, even when that was the case, the effects were generally too small for the methodology to be used as a possible future mechanism to reliably measure birds' affective state.

In theory, there are a number of possible explanations for the disappointing results of our experiments. The first is that birds had no clear preference for increased food rations and did not find social isolation and bursts of wind aversive. We find this possibility extremely unlikely. As mentioned earlier, there is evidence that food restricted birds are motivated to feed and being unable to do so is potentially frustrating (e.g. Savory et al., 1993; Hocking et al., 1996, 2001). Thus food restricted broiler breeders are expected to prefer larger food portions. Also poultry are known to be a social species that will perform distress or alarm calls when isolated from con-specifics (e.g. Applyby et al., 2004) and social isolation is used in the chick anxiety/depression model to induce a negative affective state (e.g. Kim and Sufka, 2010). Wind has also successfully been used as an aversive stimulus or as a cost in previous experiments with chickens and other species (e.g. Cabanac and Johnson, 1983; Faure and Lagadic, 1994). For example, in a consumer demand trial Faure and Lagadic (1994) used varying levels of wind speed as the cost for food deprived birds to access feed and litter. Thus there seems to be evidence that the stimuli used in this study should have been perceived as positive (CPP) or negative (CPA) to the birds tested. Also the fact that when treatment effects were statistically significant in our experiments they were always in the expected direction suggest that birds did perceive the increased food amounts as positive as intended.

The second possibility is that the methodology we used was unsuccessful but that improvements are possible that would result in establishing a CPP and/or CPA. Over the course of the three experiments we tried out different training regimes, testing times and ages, different visual cues differentiating the two pen sides, different amounts of food as the positive stimuli for CPP and different total durations of wind as the aversive stimuli for the CPA stimuli. None of these attempts resulted in a clear suggestion about the type of change in experimental design with regards to treatments that would seem more promising to measure CPP/CPA in hungry broiler breeders. Also a review of recent CPP/CPA literature does not indicate possible other changes that could have been made to improve experimental methodology (e.g. Bardo and Bevins, 2000; Tzschentke, 1998, 2007). In fact chicks in CPA studies can be successfully conditioned to avoid objects which have been associated with illness due to injection of LiCl in one session (Barber et al., 1998). Similarly, newly hatched chicks that were injected with water on one side of a CPP apparatus and with low doses of morphine (1 mg/kg) on the other formed a place preference for locations associated with the morphine after only one exposure to each side of the apparatus (Bronson et al., 1996). Our birds received much more extensive training than chickens in previous CPP/CPA studies but were apparently still unable for the most part to form strong associations between locations and positive or negative stimuli. At present we do not know how our methodology could be improved for measuring CPP/CPA in hungry broiler breeders.

Finally, the possibility exists that the lack of success is related to the fact that we worked with hungry animals and that this interfered with them forming strong associations between location and positive or negative stimuli. Broiler breeders are fed around 25–50% of what they would consume ad libitum (Savory et al., 1993) and are considered to be chronically hungry (e.g. Mench, 2002). Chronic stressors, like hunger, can impair cognition, including spatial memory, especially when the task using spatial memory is motivated by appetitive factors such as food and water (review by Conrad, 2010). This has been demonstrated in humans, with hungry children performing worse in a series of cognitive tests than children fed soon before the tests began (Cooper et al., 2011). Other stressors can have similar effects. For instance, rats that were chronically stressed by being held in restraints for 6 h per day for 21 consecutive days had impaired spatial memory in a Y-Maze task compared to non-stressed controls (Wright and Conrad, 2005).

The difficulty of training hungry broiler breeders to perform cognitive tasks has also been observed very recently in other experiments. Buckley et al. (2011) attempted to train broiler breeders kept under severe, moderate and mild levels of feed restriction to differentiate between black and white coloured arms in a Y-Maze and different sized food rewards. When the task was between food being present in one arm of the maze and no food in the other maze arm, the birds easily learned the task and showed a preference for the maze arm containing the food. However, when the birds were asked to choose between different quantities of the same high quality food in different arms of the apparatus, the severely feed restricted birds could not learn the task and the moderate to mildly restricted birds were more successful in choosing the maze arm that was associated with the larger portion of feed. It was concluded that feed restriction affected the broiler breeders learning of the task, possibly by narrowing the birds' attention so that they ignored potentially hunger-relevant contextual cues (Buckley et al., 2011).

Thus the chickens' motivation to search new areas for food, in combination with the likely effects of hunger on the birds' ability to form strong associations between visual cues and (desirable or aversive) stimuli may well have limited the effects of resource availability on the expression of pen side preferences in our experiments.

In more general terms, this paper highlights the limitations of using CPP/CPA as a welfare assessment tool. Although the majority of CPP/CPA trials have been used for drug testing purposes in pharmacology, some studies have been successful in using these methods to evaluate animal welfare. For example, de Jonge et al. (2008) used a CPP apparatus to examine piglets' preference for contra-freeloading. Piglets developed preferences to locations associated with food scattered in straw compared to locations with just straw, no food and food available in the trough. However, these piglets were housed in small groups of known individuals, were habituated to handling and did not appear to be hungry or under other forms of chronic stress. Our study demonstrates that the prospects of the successful use of such methods may be limited if subjects are experiencing chronic stress, such as that associated with hunger. Other animals affected by chronic stress, such as feed restricted sows or animals kept at high stocking densities, may well also have difficulties in learning and memory trials.

7. Conclusion

In conclusion, feed restricted broiler breeders were not successful in learning Conditioned Place Preference or Aversion tasks. They were, however, highly motivated to explore more novel environments which appears to be related to food searching. Their lack of success might also be related to a decrease in cognitive ability which is often found in chronically stressed animals. This study draws attention to the potential limitations in using CPP/CPA as a welfare assessment tool and highlights the relevance of the nutritional, housing and management conditions under which animals are kept when selecting the most appropriate methodology for animal welfare evaluations.

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