The effects of time off feed and water on the welfare of spent laying hens - Phase 2: Behavioural indicators

Final Project Report

A report for the Australian Egg Corporation Limited

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Project Title: The effects of time off feed and water on the welfare of spent laying hens - Phase 2: Behavioural indicators

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In submitting this report, the researcher has agreed to AECL publishing this material in its edited form.

Foreword

This project was conducted to investigate the welfare implications of water deprivation for different lengths of time for spent laying hens.

The acceptable time length that laying hens can spend off water before welfare is compromised is unknown. In a previous AECL project conducted by the Principle Investigators (MCCP: 2009-320), osmolality, an end measure of fluid balance regulatory systems, and other physiological measures of dehydration (packed cell volume, plasma electrolytes concentration), increased with time. However, no scientific literature exists on what can be considered acceptable changes in osmolality, or other dehydration measures, in terms of hen welfare. Interpretation could only be based on changes in humans, and clearly such interpretations are limited as the physiology of chickens significantly differs from that of humans and mammals.

Prolonged time off water ultimately leads to dehydration. Hens first try to adjust behaviourally with challenging situations. Hence, behavioural changes in situ such as increased activity due to increased searching are likely to be reliable indicators of water requirements (Experiment 1). Furthermore, hens should show an increased motivation to access water resources once their homeostasis is challenged. Hence, motivation tests could provide useful information regarding the perceived need by the hen to drink (Experiment 2).

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Acknowledgments

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Table of Contents

List of Tables

List of Figures

Abbreviations

AECL	Australian Egg Corporation Limited
FAWC	Farm Animal Welfare Council
FW	Food and water control treatment
h	Hour
LS-means	Least squares means
min	Minute
mm	Millimeter
OIE	World Organisation for Animal Health ('Organisation International des Epizooties')
PCV	Packed cell volume
s	Second
SARDI	South Australian Research and Development Institute
SEM	Standard error of the mean
W	Water control treatment, off feed for 32h

Executive Summary

The 'Australian Standards and Guidelines for the Welfare of Animals — Land Transport of Livestock' (Animal Health Australia, 2012) states that the maximum time off water for poultry during transport should not exceed 24h (item SB10.1). However, there is no scientific evidence to indicate the suitability of this recommendation in terms of hen welfare. In a previous AECL project conducted by some of the principle investigators (MCCP: 2009-320), osmolality and other physiological indicators of dehydration (packed cell volume and plasma electrolyte concentration) increased as time off feed and water increased. However, no scientific literature exists on what can be considered acceptable changes in osmolality, or other dehydration measures, in terms of hen welfare. The present experiment aimed to equate physiological changes induced by time off water with behavioural changes in order to assess its welfare implications.

Prolonged time off water ultimately leads to dehydration. Hens first try to adjust behaviourally to challenging situations. Hence, behavioural changes in situ such as increased activity due to increased searching are likely to be reliable indicators of water requirements. Alternatively, if that search is unsuccessful, hens usually start performing abnormal behaviours such as redirected and displacement behaviours, for example excessive preening, pacing, or aggression toward conspecifics. Ultimately, if these behavioural changes are unsuccessful in attaining water, the hens become physiologically compromised and may enter a state of reduced activity and reduced responsiveness to startling stimuli ('lethargy'). Hence, Experiment 1 investigated the behavioural changes occurring at 12, 18, 24 or 32h after water and feed removal, or solely after 32h off feed, in cages of 5 hens (9 cages per treatment), in conjunction with the physiological measures of corticosterone concentration, packed cell volume, osmolality, comb colour score, and weight loss. Experiment 1 showed that behavioural changes occurred over the first 12h (first time point) and 18h, suggesting that this is a period during which hens adjust their behaviour in response to the thwarting situation. These behavioural changes preceded the physiological changes at 24h (weight loss) and 32h (packed cell volume, osmolality). However, the reduced activity ('lethargic state') that we predicted as time off water and feed increased did not eventuate.

Since the demand for water is inelastic in most animals, motivation should be high to work for water. Squeezing through narrow openings has previously been validated in laying hens to assess the level of motivation to access a resource and in turn the importance of the environmental resource. Hence, Experiment 2 employed a motivation test using the rationale that higher dehydration times should lead to a higher price paid to access water, in this case willingness to squeeze through a narrow opening. Twenty hens were subjected to water removal for various lengths of time (0, 12, 18, 24 or 32h) and work level with door gaps from wide to narrow (150, 135, 120 and 100mm) following an incomplete randomised block design with 10 tests per hen across 5 weeks. The results showed that the use of narrow vertical door gaps had little effect as a measure of the motivation of the hens to reach the water drinker located in the adjacent side of the testing apparatus. Nonetheless, clear behavioural differences appeared as a result of the length of water removal, reaching a plateau at 24h with no differences between 24h and 32h in most behaviours (e.g. drinking duration). However, changes were already seen in some behaviours at 18h after water removal (e.g. location of the hen close to the drinker, reduced standing).

Overall Conclusions

The present experiment aimed to equate physiological changes induced by time off water with behavioural changes in order to assess its welfare implications. A previous AECL project (MCCP: 2009-320) provided physiological evidence that, under favourable handling and climatic conditions, the welfare of spent hens is challenged by deprivation of food and water for 24h and more, using the time points of 12, 24, 28 and 32h. The present project attempted to go further by looking at behavioural evidence in addition to physiological evidence, including 18h as a time point instead of 28h, and adding a control treatment given ad libitum access to water but no feed for 32h.

The results indicate that, under favourable handling, social and climatic conditions, the welfare of spent hens is challenged due to water deprivation. Experiment 1 showed that behavioural changes occurred as early as 12h and 18h, suggesting that this is a period during which hens adjusted their behaviour in response to the thwarting situation and which preceded the physiological changes seen at 24h and 32h. Experiment 2 showed clear behavioural differences as a result of the length of water removal, reaching a plateau at 24h with no differences between 24h and 32h on most behaviours (e.g. drinking duration). However, changes were already apparent for some behaviours after 18h of water removal (e.g. location of the hen close to the drinker, reduced standing).

In conclusion, hens changed their behaviour as early as 12h after water deprivation (first time point). Nevertheless, behavioural changes do not necessary equate strictly to a state of compromised welfare, as behaviour is primarily a coping strategy to adapt to change. Physiological changes occurred by 24h, to a similar level to what was seen at 32h, which suggests that a plateau was reached in terms of acute physiological adaptation. Consequently, the results presented in this report, in accordance with our previous report (MCCP: 2009-320), questions the welfare of hens that have water withdrawn for 24h or longer. Nevertheless, there are no clearly defined thresholds indicative of acceptable and unacceptable welfare in the measured responses. When relying on behavioral, physiological, and fitness measures to determine welfare risks, a judgment is made about what degree of change in these indicators is likely to indicate compromised animal welfare. If one favours a conservative decision, the behavioural changes suggested that welfare starts being compromised earlier than 24h after water removal, and probably somewhere between 18h and 24h. However, if one favours the physiological changes, physiological adaptation reached a plateau at 24h, suggesting that 24h appear as the maximum acceptable time off water and that 32h is too long.

These experiments have been conducted under favourable handling and climatic conditions. It should be recognized that factors other than feed and water deprivation are likely to influence hen welfare during transport, such as the health status of the hens prior to loading, their body condition, stress of handling, social stress of mixing, duration of transport and the weather during transport and lairage. Further research is required to determine what factors specifically influence the welfare of spent hens during transport in field conditions.

1 Literature review: The welfare implications of water and feed deprivation for laying hens

1.1 Hen welfare

Animal welfare can be defined as "how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare if (as indicated by scientific evidence) it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and not suffering from unpleasant states such as pain, fear, and distress. Good animal welfare requires disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling and humane slaughter/killing. 'Animal welfare' refers to the state of the animal; the treatment that an animal receives is covered by other terms such as animal care, animal husbandry, and humane treatment" (OIE, 2010).

This definition covers quite comprehensively all aspects that can impact on the welfare of an animal. Nevertheless, assessing animal welfare on-farm remains practically challenging. The assessment of animal welfare requires the use of multiple indicators from multiple disciplines but their relative importance has yet to be clarified. Furthermore there are no clearly defined thresholds indicative of acceptable and unacceptable welfare in the measured responses. Thus, interpreting the welfare implications of particular situation is problematic. When relying on behavioral, physiological, and fitness measures to determine welfare risks, a judgment is made about what degree of change in these indicators is likely to indicate compromised animal welfare.

The Five Freedoms (FAWC, 1979) provides a general framework that has been widely accepted among welfare scientists for tackling core welfare components (although it does not specify thresholds indicative of acceptable and unacceptable welfare). This specifies that an animal is in a good state of welfare if it is free from hunger and thirst; discomfort; pain, injury and disease; free to express normal behaviour; and free from fear and distress.

Free from hunger and thirst comes as the most basic of these Freedoms. Nevertheless, there is still a lack of understanding of the exact implication for welfare when an animal cannot access feed and water, and particularly of the length of time after which welfare can be considered compromised.

1.2 Welfare implications of feed and water restriction

A key issue for poultry transport standards is the time that hens are without water. This is reflected in the first standard defined in the 'Australian Standards and Guidelines for the Welfare of Animals — Land Transport of Livestock', which states that the maximum time off water for poultry during transport should not exceed 24h (item SB10.1, Animal Health Australia, 2012). Although the period pre-slaughter is not specified in the case of laying hens, by default it implies a total of 24h from loading to slaughter. However, there is no scientific evidence to indicate the suitability of this recommendation in terms of hen welfare.

A previous project was commissioned by AECL (MCCP: 2009-320) to determine the effects of time off feed and water on the physiology of spent laying hens. The physiological effects of time off feed and water for up to 32h in spent laying hens was examined with blood samples collected at 12, 24, 28 and 32h. Treatment hens were placed in groups of 10 in transport crates at 0h without feed or water. In contrast, Control hens remained in their accommodation cages and were provided with feed and water ad libitum. All hens were housed in an

environmentally-controlled laying shed at a large commercial farm and kept between 21-24ºC. This design in an environmentally-controlled facility was used because of the difficulty of studying feed and water withdrawal under conditions of transport. Indices of dehydration (osmolality, packed cell volume and plasma electrolyte concentration), metabolic challenge (plasma glucose and lactate concentrations) and stress physiology (plasma corticosterone concentration) were studied.

The most pertinent finding from this previous experiment in relation to hen welfare was the effect on osmolality. In comparison to Control hens sampled at the same time, osmolality increased by +2% after 12h of feed and water withdrawal, and by +7, +6 and +7% at 24, 28 and 32h respectively. Osmolality is a sensitive and widely accepted end-measure of fluid balance regulatory systems (Hatton *et al.*, 1970; Szczepanska-Sadowska *et al.*, 1984; McKenna & Thompson, 1998). It is a measure of the concentration of solutes in the extracellular fluid, expressed as moles per kilogram of water, which increases following loss of body fluid (Chloe and Strange, 2009). Plasma osmolality has previously been reported to provide a sensitive measure of dehydration in poultry. Other studies using pullets reported increase of +3%, +5%, +7.9% and +8.2% by 24, 48, 72 and 96h when only water deprived (Koike *et al.*, 1983) and by +7% after 24h of food and water deprivation (Koike *et al.*, 1977), a value identical to ours when both feed and water were withdrawn. Osmolality has also been reported to increase in broiler chickens following 24h (Arad *et al.*, 1985) and 48h (Zhou *et al.*, 1999) of water deprivation (+10% and +9%, respectively), when compared to each bird's baseline osmolality before the challenge. However Knowles *et al.* (1995) found that the plasma osmolality of broilers only increased by +0.03% at 17°C and +1.3% at 23°C with deprivation of feed and water for 24h when compared to unrestricted Controls, a result difficult to explain in relation to the rest of the literature which suggests larger increases. Note that a reason for the discrepancy may lie in the method of measurement, variations in feed intake, ambient conditions or different initial physiological states or production stages.

Although previous experiments provided useful data on the effects of time off water on physiological variables, these results do not allow us to reach conclusions regarding the welfare implications of this practice. There is a crucial need to equate those physiological changes with the discomfort or pain that may be experienced by the hens at various degrees of dehydration. In humans, the initial symptoms of dehydration occur when osmolality increase by about 2-2.5%, become serious and painful by 5%, and can be fatal when it reaches 10% or more (Jequier and Constant, 2010). However, no similar quantification of the symptoms of dehydration with osmolality, or other dehydration measures, exists in the scientific literature in laying hens. Thus, interpretation of these data in terms of hen welfare is clearly limited. Further research is necessary to interpret these physiological indicators of dehydration by equating these with behavioural measures indicative of the hen's perception of those states of dehydration.

1.3 Behavioural changes

In thwarting situations, behavioural changes in situ normally occur, such as increased activity or locomotion (due to increased searching for the desired resource), vocalizations and panting. Furthermore, so-called abnormal behaviours can appear if conflict or thwarting conditions persist in the longer term, such as redirected behaviours and displacement activities when hens are feed or water deprived (Duncan & Wood-Gush, 1972; Haskell *et al.*, 2000). Abnormal behaviours in this situation may include aggression, pacing, excessive drinker manipulation, head flicks, etc. These behaviours are characteristics as being displayed out of context. Ultimately, if these behavioural changes are unsuccessful to attain water, the hens become physiologically compromised and enter a state of reduced activity and reduced responsiveness to startling stimuli (i.e. 'lethargy'). Behaviour represents one of the most robust outputs of an animal's perception. Yet, a behavioural approach such as this has not been used to assess the welfare implications of the length of time off water for laying hens.

1.4 Motivation tests

Prolonged time off water ultimately results in dehydration. Hens initially demonstrate an increased motivation to access water. This implies that behavioural demand tests (also called 'motivation tests') could provide useful information regarding the perceived need by the hen to drink. This methodology is widely accepted by animal welfare scientists in order to assess the importance of a particular resource for the animal (Kirkden & Pajor, 2006; Jensen & Pedersen, 2008), and has been previously validated to assess the welfare implications of a nest for laying hens (Cooper & Appleby, 1996). It typically uses measures of the amount of work that an animal will perform to obtain the resource, with the performance of high workloads interpreted as a high need for that resource in thwarting situations. That is, hens that are experiencing greater need to drink will work harder to obtain access to water than hens with lesser need to drink, thus providing a quantifiable measure of this motivation.

1.5 Aims of the current research project

This project investigated the effects of length of time off water on the behaviour of laying hens in order to assess its implications in terms of welfare. In the previous AECL project (MCCP: 2009-320), treatment hens were deprived of both water and feed whereas control hens had access to water and feed ad libitum. This project more comprehensively aimed to examine this topic by using two control treatments: one with access to water and feed ad libitum and another one with access to water but no access to feed. Hence, this design allowed the dissociation of the effects of feed and water deprivation from the effects of water deprivation only. Although transport conditions (e.g. handling, changes in ambient temperature and humidity) can affect water requirements, these were not considered in this project, as they would require a much larger sample size and number of treatments. Nonetheless, these effects should be considered in a follow up project on the effects of transport in commercial conditions on the behaviour, physiology and meat quality of spent hens by observing the hens in various transport conditions.

1.6 Hypotheses of the current research project

Prolonged time off water ultimately leads to dehydration. Hens first try to adjust behaviourally. Hence, behavioural changes in situ such as increased activity due to increased searching are likely to be reliable indicators of water requirements (Experiment 1).

Furthermore, hens should show an increased motivation to access water resources once their homeostasis is threatened. Hence, motivation tests could provide useful information regarding the perceived need of the hen to drink (Experiment 2).

2 Experiment 1: Behavioural changes induced by water and feed withdrawal

2.1 Rationale

When access to a needed resource is restricted, domestic hens usually intensify specific behaviours that were previously successful in gaining access to that resource, such as pecking the nipple drinker to obtain water. If that behaviour is unsuccessful, domestic hens usually start performing abnormal behaviours such as redirected and displacement behaviours, for example excessive preening, pacing, or aggression toward conspecifics (Duncan & Wood-Gush, 1972; Haskell *et al.*, 2000). These so-called abnormal behaviours are usually characterized by an increase in frequency and duration of the behaviour or by the behaviour occurring out of context. Identifying the type and frequency of these behaviours should help to quantify the perception of the situation by the hen.

Our initial prediction was that as dehydration levels increase, the behavioural reaction of the hens toward an empty nipple drinker, such as the pecking rate, should increase. Alternatively, the frequency of other behaviour may increase (higher 'behavioural switching') with a higher occurrence of activities such as pacing, aggression, or preening, followed by reduced activity and reduced responsiveness to startling stimuli ('lethargy').

2.2 Methods

2.2.1 Housing

The project was approved by the Victorian Department of Environment and Primary Industries (application number 39.12) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Two hundred and seventy, 81 week-old, Hy-line Brown spent laying hens were obtained from a cage commercial farm and transported to the Scolexia Animal Research Facility, in Attwood, Victoria. For transport, hens were placed 10 per crate by mixing 2 initial cages in order to minimize hierarchy disruption when allocated to their new cages at arrival. Hens were housed in groups of 5, with a space allowance of 550cm^2 per hen, in a three-tier conventional cage system on 2 sides of a row in 1 shed, and given 2 weeks to acclimatise to their new environment prior to the start of the test. The hens were on a 15h light schedule (0530h-2030h) and the temperature was always maintained between 19.2 and 24.2°C with a relative humidity of 50-60%. However, lights were turned on manually, at 0624h for replicate 1 and 0417h for replicate 2.

2.2.2 Treatments

Cages were randomly allocated over 2 replicates to 1 of 6 treatments (n=9 cages per treatment): 12h, 18h, 24h or 32h off water and feed, ad libitum access to water and feed ('FW control'), or ad libitum access to water but 32h off feed ('W control'). Replicates were conducted by submitting 1 side of the cage row to the test and the other side 48h later after the first replicate was completed. For each replicate, treatments started at different times of the day but finished at the same time of the day at 1500h (**Figure 2-1**). Hens were maintained off water by turning off the water lines and blocking access to the nipple drinker by covering it with a PVC partition in the back of the cage. Feed was removed manually at the start of each treatment by using a portable vaccum cleaner. One control group was provided with undisturbed access to water and feed ad libitum ('FW control') whereas another control group was provided with undisturbed access to water but no access to feed for 32h ('W control').

These control groups allowed monitoring of the normal circadian rhythm in behaviour and physiology of hens.
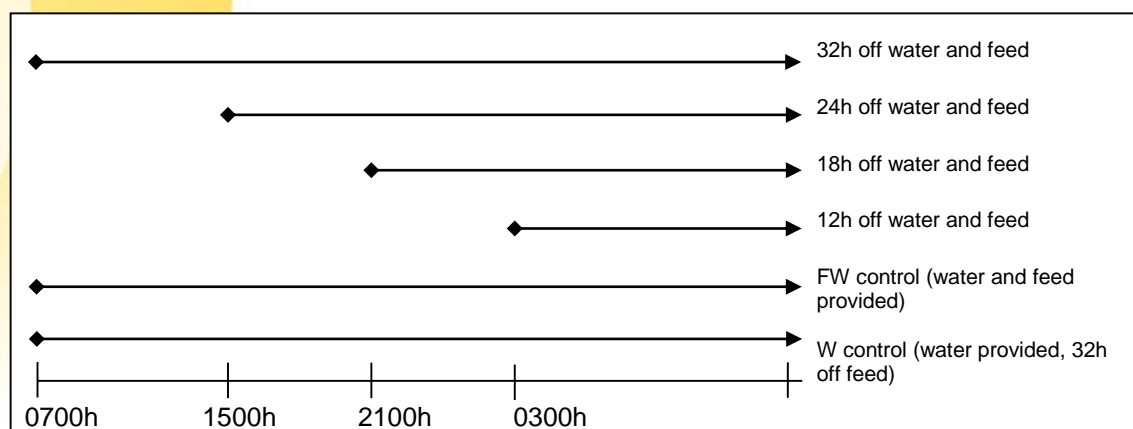
Figure 2-1: Experimental design of the treatment groups overtime in Experiment 1

2.2.3 Data collection

2.2.3.1 Behaviour

Each cage was equipped with a front camera that continuously recorded all hens in the cage for the whole duration of the test. Behaviour was analysed for the last 12h for each treatment using a 3-min scan sampling method to record the number of hens with their head up, with their head in the feeder, with their head out of the front horizontal bars of the cage, inactive, or not visible, according to an ethogram (**Table 2-1**). Behaviours were scored for each cage of 5 hens without attempting to identify individual hens within the cage. Unfortunately, we could not elaborate on a more detailed ethogram to record other behaviour of interest (e.g. pecking at feeder, empty drinker or conspecifics, preening, pacing) as initially intended due to the space allowance of 550 cm^2 per hen, low quality of the images obtained in the commercial-like cage setting and with low light leading to low visibility of the hens' full body from the front view. Hence, only behaviour observations based on the visible upper part of the hens (head and neck) were possible. Behaviour was analysed by 2 observers, each observer assigned to 1 of the 2 replicates, with an inter-observer reliability superior to 90% agreement.

Table 2-1: Ethogram used for Experiment 1

Behaviour*	Description
Head up	Standing with head visible and above dorsal surface of body
Head out	At least one third of the head extended through bars at the front of the cage. The head is not in the feeder but above it.
Head in feeder	Head below dorsal surface of body and positioned through bars and at least one third of head in the feeder at the front of cage.
Inactive	Immobile, with head below dorsal surface of body, can be standing or sitting. Hen inactive, not feeding, head out, preening nor interacting with conspecifics or cage fixtures.
Not visible	Below the visible top part of the cage or in the back of the cage.

*All behaviours were mutually exclusive

2.2.3.2 Physiology, live weight and comb colour score

At the end of the withdrawal period (1500h), 4 random hens (n=36 hens per treatment) from each cage were removed at the same time. A blood sample (2mL) was collected from the wing vein in a lithium-heparin tube and the hen was weighed. Therefore, blood samples were collected at the same time of day for all treatments in order to control for circadian effects. A single observer, blind to treatments, scored their comb colour from 1 to 7, using the 'comb colour scale' from the Bristol Welfare Assurance Programme hen assessment (Leeb *et al.*, 2005). To avoid disturbing cages prior to sampling, and due to the large number of hens to be sampled, we adopted a blood sampling schedule where 4 teams of 2 people simultaneously sampled all hens from 1 cage, moving along 1 tier from the front to the back of the row (cage numbers 1 to 9). An interval of 30 min was then imposed before sampling cages from the middle tier in the same order, and another interval of 30 min before sampling cages from the top tier. All cages pertaining to the same replicate were located on the same side. The time to blood sample each hen was recorded if it exceeded 2 min. Blood samples were analysed within the next hour on-site for packed cell volume collection using a purpose built centrifuge. The remainder of the blood sample was subsequently centrifuged on-site, stored at -20°C, and analysed for osmolality using an Osmometer (Advanced® Micro-Osmometer Model 332, Advanced Instruments, INC), performed by an external diagnostic lab (Sullivan Nicolaides Pathology, Brisbane, QLD). Corticosterone concentrations were analysed using a radioimmunoassay developed in-house after hexane extraction, according to a previously validated protocol (Etches, 1976; Downing & Bryden, 2008). The number of eggs laid per cage was recorded 4 days prior to the start of the test to account for the proportion of layers per cage in each treatment.

2.2.4 Statistical analysis

Only 10 full hours of videos could be analysed (0330h-1330h) out of the last 12h of treatment because the last 90min was sometimes disrupted by handling and blood sampling which occurred from 1330 to 1630h, with a median value of 1500h corresponding to the end of the treatment period. The behavioural data were pooled by hour, from the first to the tenth hour of observation, prior to analysis. Three cages were lost due to technical issues with the cameras. Out of the 510 scan observations collected, hours with less than half the scans were discarded, which occurred for 8.6% of all observations (44 out of 510 scans) due to poor visibility, camera displacement or human disruption.

All data met the criteria for normality and homogeneity of variance, except corticosterone concentration, which had to be log-transformed. Data were analysed using a mixed model (Proc Mixed, SAS Inst. Inc., Cary, NC, USA). For behaviour, the model included treatment, hour of observation, replicate, and the interactions of treatment × hour of observation and treatment × replicate as fixed effects, and cage was included as a random effect. For corticosterone concentration, osmolality, packed cell volume, comb colour and weight loss, the model included treatment, replicate and their interaction as fixed effects, and bird was included as a random effect. For live weight, the same model was used except that we added the fixed effect of initial live weight of the cage at arrival, as an average of the 5 birds. When significant differences ($P < 0.05$) were detected, Tukey–Kramer adjustments were used to account for the number of pairwise comparisons between treatments. Data are presented as LS-means ± SEM.

2.3 Results

Replicate 1 had to be repeated due to one of the personnel feeding all hens halfway through the treatment on the morning prior to data collection. Treatment allocation was randomised again across all cages for Replicate 1, and the birds given 1 week prior to the new treatment imposition. However, the results suggested that this was not sufficient because the previous

attempted treatment, when included in the model for the (new) Replicate 1 either as a main effect or with its interaction with the new treatment, significantly affected the corticosterone concentration, comb colour, osmolality and several behaviours (head out of the cage, feeding, and inactivity) in the new Replicate 1 (Appendixes 1 and 2). Hence, it was decided to discard the data for Replicate 1 from the analyses since the interpretation of these results would require consideration of 25 combinations of treatments (5 previous treatments × 5 new treatments), with ultimately too small sample sizes to derive meaningful interpretations. Hence, results are only presented for Replicate 2. Data for Replicate 1 are presented in Appendixes 1 and 2.

2.3.1 Behaviour

All 10 hours of behavioural observations included in the analysis were during the light schedule apart from the first 47min of the first hour of observation.

Head out varied according to treatment (P = 0.006; **Figure 2-2** and **Table 2-2**), with hens in the 18h off water and feed treatment displaying more head out of the cage, through the bars, than hens in the W control treatment, for 32h off feed (P = 0.002). Head out also varied according to the hour of observation (P < 0.0001), and the interaction between treatment and the hour of observation was significant (P = 0.05; Appendix 3). However, the interaction is overly complex to interpret due to the large number of post-hoc comparisons (5 treatments × 10h of observation). Head out also varied according to the tier (P = 0.003), with cages in the top tier displaying less head out of the cage (1.8 ± 0.8%) than the middle or bottom tiers (5.1 ± 0.8%, P = 0.005; and 5.0 ± 1.0%, P = 0.03, respectively).
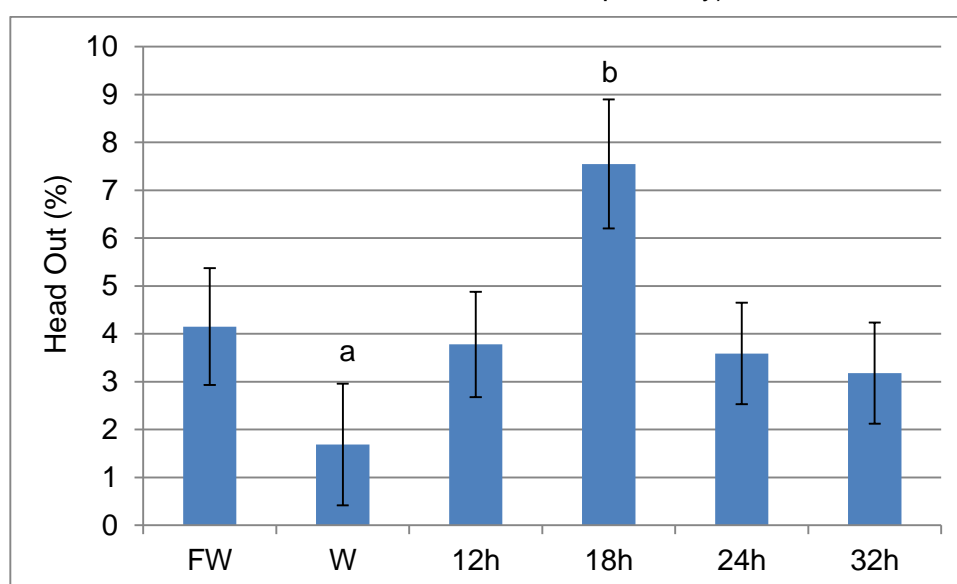
Note: Means with different superscripts ([a-b]) differ significantly (P < 0.05).

Figure 2-2: Head out behaviour (LS-means ± SEM) by treatment for Replicate 2

Head up varied according to treatment (P = 0.006; **Figure 2-3** and **Table 2-2**), with hens in the 12h and 24h treatments displaying more head up than hens in the W control treatment or hens in the 18h treatment (all P ≤ 0.05). Head out also varied according to the hour of observation (P < 0.0001; Appendix 3), but the interaction between treatment and hour of observation was not significant (P = 0.08).
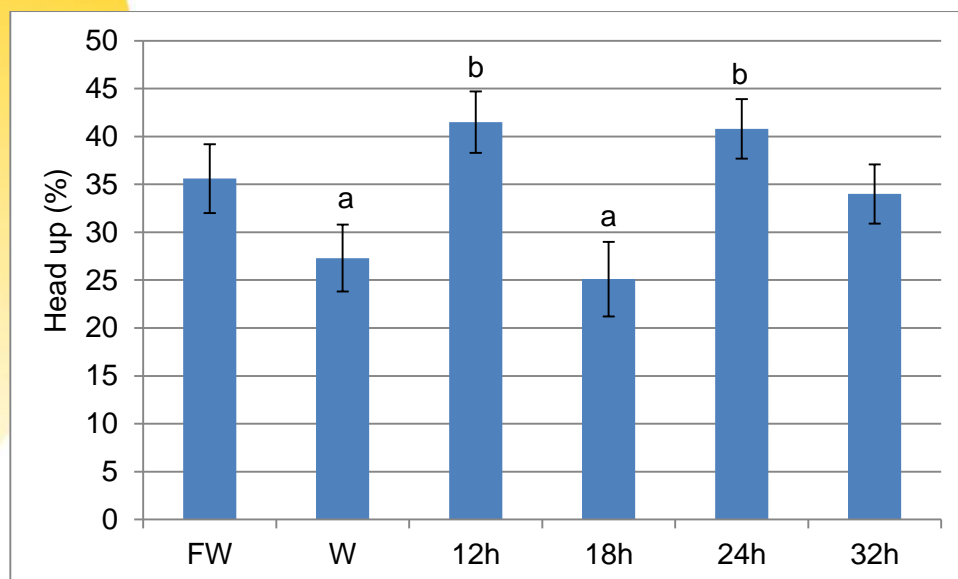
Note: Means with different superscripts (ᵃ⁻ᵇ) differ significantly (P < 0.05).

Figure 2-3: Head up behaviour (LS-means ± SEM) by treatment for Replicate 2

Head in the feeder varied according to treatment (P < 0.0001; **Figure 2-4** and **Table 2-2**), with hens in the 12h treatment displaying more head in the feeder than hens in the W control treatment or those in the 24h or 32h treatments (all P ≤ 0.04). Hens in the 18h off treatment also displayed more head in the feeder than hens in the W control treatment off feed (P = 0.0009). Head out also varied according to the hour of observation (P = 0.0003; Appendix 3), and the interaction between treatment and hour of observation was significant (P = 0.01).
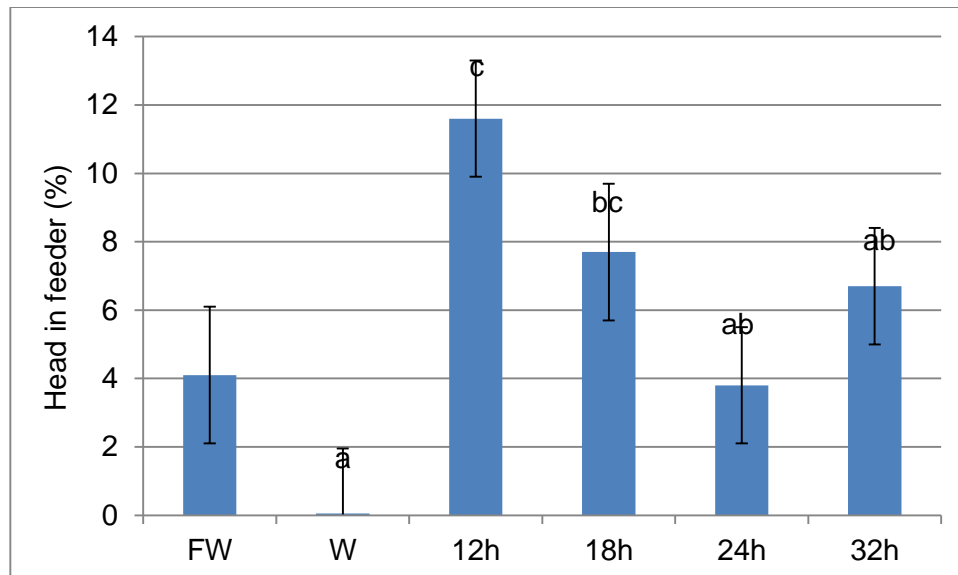
Note: Means with different superscripts (ᵃ⁻ᶜ) differ significantly (P < 0.05).

Figure 2-4: Head in feeder (LS-means ± SEM) by treatment for Replicate 2

The proportion of hens inactive varied according to treatment (P = 0.0002; **Figure 2-5** and **Table 2-2**), with hens in the 18h treatment spending more time inactive than hens in the FW control treatment or hens in the 12h treatment (P = 0.02 and P = 0.006, respectively). Hens in the W control treatment, off feed for 32h, also spent more time inactive than hens in the FW, 12h or 24h treatments (all P ≤ 0.02). Head out also varied according to the hour of

observation (P < 0.0001; Appendix 3), but the interaction between treatment and hour of observation was not significant (P = 0.11).
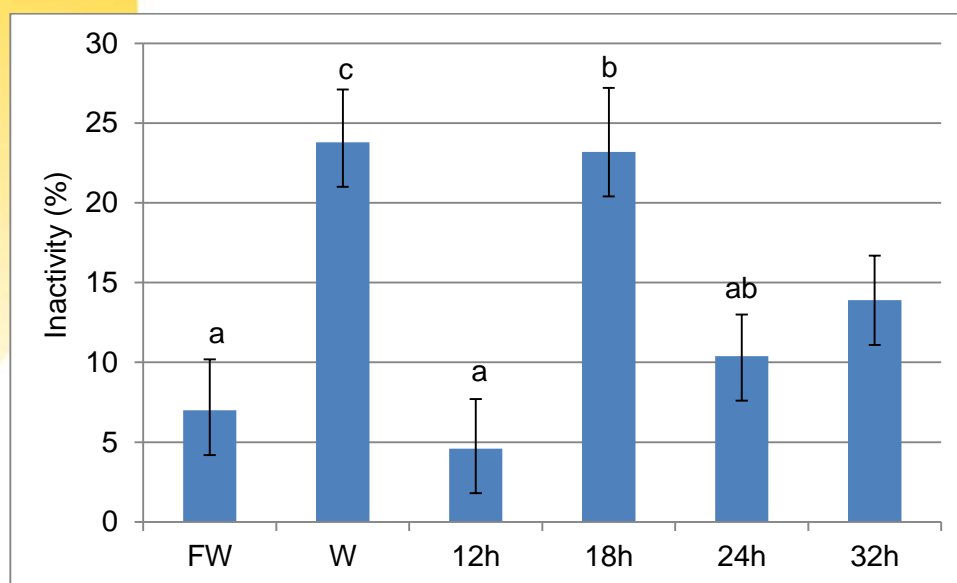
Means with different superscripts ($^{a-c}$) differ significantly (P < 0.05).

Figure 2-5: Inactive behaviour (LS-means ± SEM) by treatment for Replicate 2

The proportion of observations classified as not visible (overall mean ± SEM : 41 ± 0.9%) varied according to treatment (P = 0.006; **Table 2-2**), with less hens visible in the W off feed control treatment as compared to hens in the 18h treatment (50.6 ± 5.5% vs. 35.5 ± 5.8%, P = 0.01). The proportion of hens not visible also varied according to the hour of observation (P < 0.0001; Appendix 3) but the interaction between treatment and hour of observation was not significant (P = 0.32). Although this overall frequency of this variable "not visible" appears high (41% of observations), it should be noted that 1 hen not visible in the cage of 5 hens lead to a 20% of not visible observations. Hence, these results suggest that, on average, 2 out of the 5 hens in the cage were not visible during the observations.

2.3.2 Physiology, weight and comb colour score

2.3.2.1 Live weight and weight loss

Treatments were randomly allocated within each replicate, and the initial live weight for the cage at arrival or the number of eggs laid per cage after the 2 week of habituation did not differ according to treatment (P = 0.18 and P = 0.82, respectively).

Live weight did not vary according to treatment (P = 0.13; **Table 2-3**). The initial live weight for the cage at arrival was included as a covariate in the model but was not significant (P = 0.26).

When accounting for the initial live weight for the cage at arrival, as an average of the 5 birds, weight loss varied according to treatment (P < 0.0001; **Figure 2-6**). Hens in the 12h, 24h or 32h off water and feed treatments or solely off feed for 32h (W control) lost more weight than hens in the FW control treatment (all P < 0.04). In comparison to hens in the FW control treatment which gained 4.6% of their body weight since arrival, hens in the 12h, 18h, 24h, or 32h treatments lost 4.1, 3.5, 9.9 and 8.2 % of their initial weight at arrival, respectively, and hens in the 32h off feed (W control) lost 8.4% of their initial weight at arrival. The statistical power of this test was 0.3.

Table 2-2: Behaviours by treatment (LS-means ± SEM) for Replicate 2

Variables (%)	FW (0h)	W (32h F)	12h	18h	24h	32h	Treatment (T) P-value	Hour (H) P-value	T x H P-value
Head out	4.1 ± 1.2	1.7 ± 1.3^a	3.8 ± 1.1	7.6 ± 1.4^b	3.6 ± 1.1	3.2 ± 1.1	**0.006**	**<0.0001**	**0.05**
Head up	35.6 ± 3.6	27.3 ± 3.5^a	41.5 ± 3.2^b	25.1 ± 3.9^a	40.8 ± 3.1^b	34.0 ± 3.1	**0.006**	**<0.0001**	0.08
Head in feeder	4.1 ± 2.0	0.1 ± 1.9^a	11.6 ± 1.7^c	7.7 ± 2.1^{bc}	3.8 ± 1.7^{ab}	6.7 ± 1.7^{ab}	**<0.0001**	**0.0003**	**0.01**
Inactive	7.0 ± 3.2^a	23.8 ± 3.3^c	4.6 ± 3.1^a	23.2 ± 4.1^b	10.4 ± 2.7^{ab}	14.0 ± 2.8	**0.0002**	**<0.0001**	0.11
Not visible	51.4 ± 5.9	50.6 ± 5.5^a	36.9 ± 4.9	35.5 ± 5.8^b	41.0 ± 5.1	44.7 ± 4.8	**0.006**	**<0.001**	0.32

Significant P-values are highlighted in bold. Means with different superscripts (a-c) differ significantly between columns (P < 0.05).

Table 2-3: Physiology, weight and comb colour score by treatment (LS-means ± SEM) for Replicate 2

Variables (%)	FW (0h)	W (32h F)	12h	18h	24h	32h	Treatment (T) P-value
Cage average weight at arrival (g)	2045 ± 59	2194 ± 53	2176 ± 53	2098 ± 59	2232 ± 53	2083 ± 59	0.18
Live weight (g)	2231 ± 79	2097 ± 84	2018 ± 95	2020 ± 111	1983 ± 95	1925 ± 74	0.13
Weight loss (g)	-95 ± 49^a	184 ± 44^b	89 ± 44^b	73 ± 49	222 ± 44^b	170 ± 49^b	**<0.0001**
Corticosterone (ng/mL)*	2.4 ± 0.5^a	2.2 ± 0.4^a	4.4 ± 0.4^b	5.7 ± 0.5^c	3.8 ± 0.4^{ab}	2.7 ± 0.5^a	**<0.0001**
Packed cell volume (%)	27.9 ± 0.7^a	32.2 ± 0.7^b	29.9 ± 0.7	30.5 ± 0.7	30.5 ± 0.7^b	31.5 ± 0.5	**0.002**
Osmolality (mosmol/kg)	332 ± 2^b	325 ± 2^a	334 ± 2^b	330 ± 2^{ab}	333 ± 2^b	342 ± 2^c	**<0.0001**
Comb colour score	5.2 ± 0.2	4.9 ± 0.2	4.7 ± 0.2	4.6 ± 0.2	4.8 ± 0.2	5.3 ± 0.2	0.22

Corticosterone concentration is presented as untransformed means but was analysed using a log transformation.

Significant P-values are highlighted in bold. Means with different superscripts (a-c) differ significantly between columns (P < 0.05).
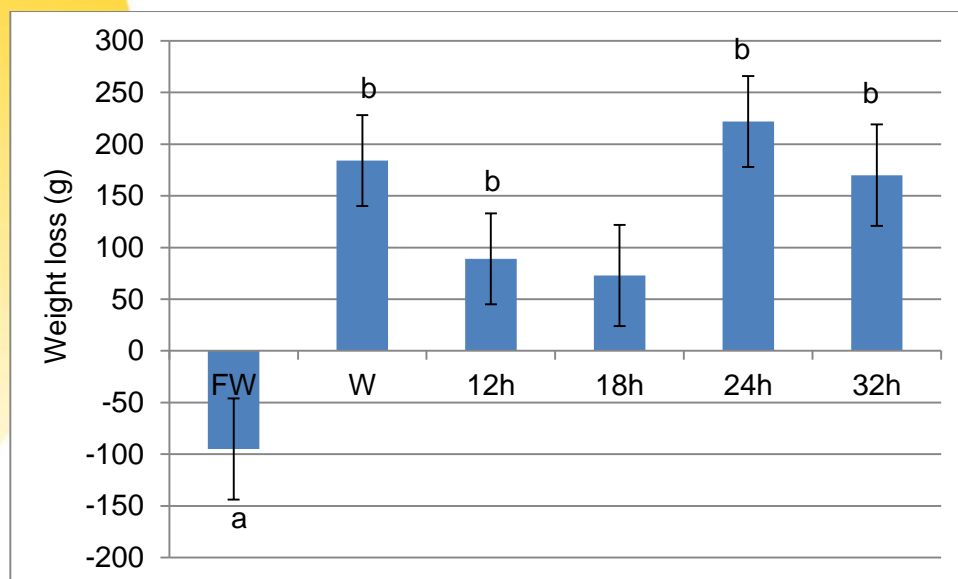
Means within replicate with different superscripts (a-b) differ significantly (P < 0.05).

Figure 2-6: Weight loss (LS-means ± SEM) by treatment for Replicate 2

2.3.2.2 Corticosterone concentration

Corticosterone concentration varied according to treatment ($P < 0.0001$; **Figure 2-7** and **Figure 2-8**). Hens in the 12h or 18h treatments had higher corticosterone concentrations than either FW or W control treatments (all $P < 0.01$) but hens in the 24h treatment had lower corticosterone concentrations than those in the 18h treatment ($P = 0.04$) and hens in the 32h treatment had lower corticosterone concentrations than both 18h and 12h treatments ($P = 0.0004$ and $P = 0.05$, respectively). The statistical power of this test was 0.6.

When added as covariates, the time to blood sample, recorded if it exceeded 2 min, the order of sampling, the tier, or the position of the cage in the row did not have any effect on corticosterone concentration (all $P > 0.1$).
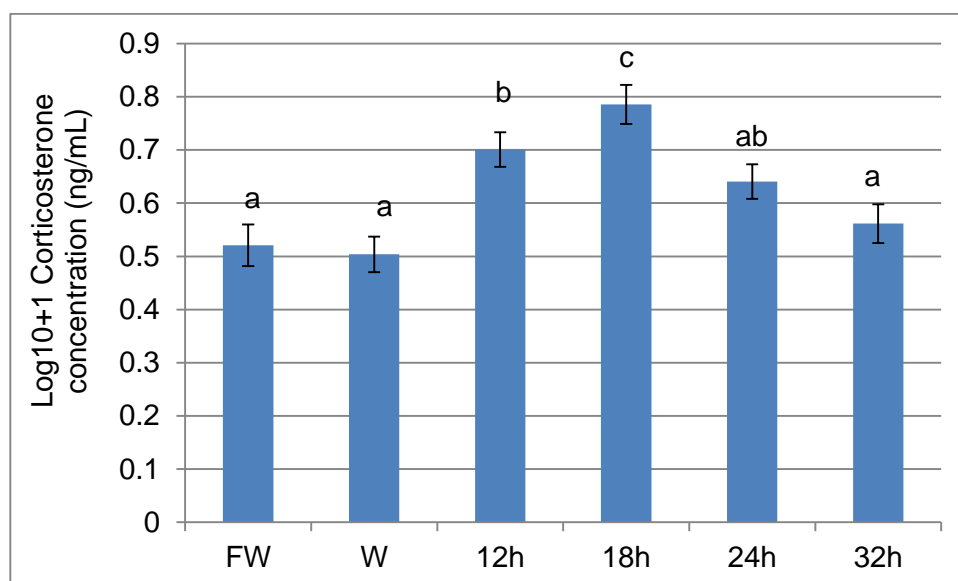
Means within replicate with different superscripts (a-c) differ significantly (P < 0.05).

Figure 2-7: Log transformed Corticosterone concentration (LS-means ± SEM) by treatment for Replicate 2
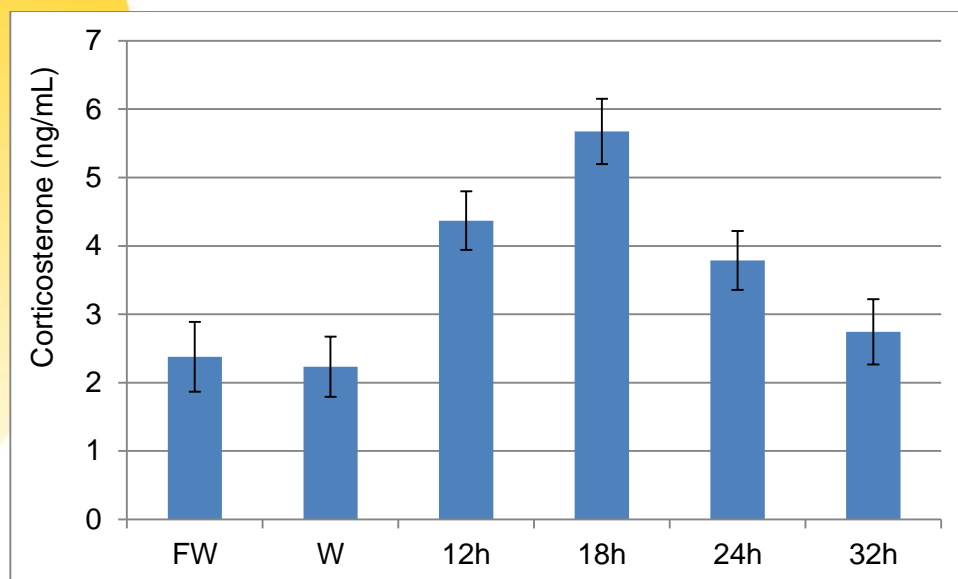
Figure 2-8: Untransformed corticosterone concentration (LS-means ± SEM) by treatment for Replicate 2

2.3.2.3   Packed cell volume

Packed cell volume (PCV) varied according to treatment (P = 0.002; **Figure 2-9**). Hens in the 32h treatment and those in the W control treatment, off feed for 32h, showed an increase of 12.9% and 15.4% in PCV, respectively, compared to hens in the FW control treatment (P = 0.01 and P = 0.0006, respectively). The statistical power of this test was 0.3.

2.3.2.4   Osmolality

Osmolality varied according to treatment (P < 0.0001; **Figure 2-10**). Hens in the 32h treatment had higher osmolality compared to hens in the 24h, 18h and 12h treatments (all P ≤ 0.02), and 12h and 24h in turn had higher osmolality than hens in the W control treatment (both P ≤ 0.03). However, hens in the FW control treatment had higher osmolality than hens in the W control treatment, off feed for 32h (P = 0.05). The statistical power of this test was 0.4.

2.3.2.5   Comb colour score

Comb colour score did not vary according to treatment (P = 0.22, **Table 2-3**). The statistical power of this test was 0.1.
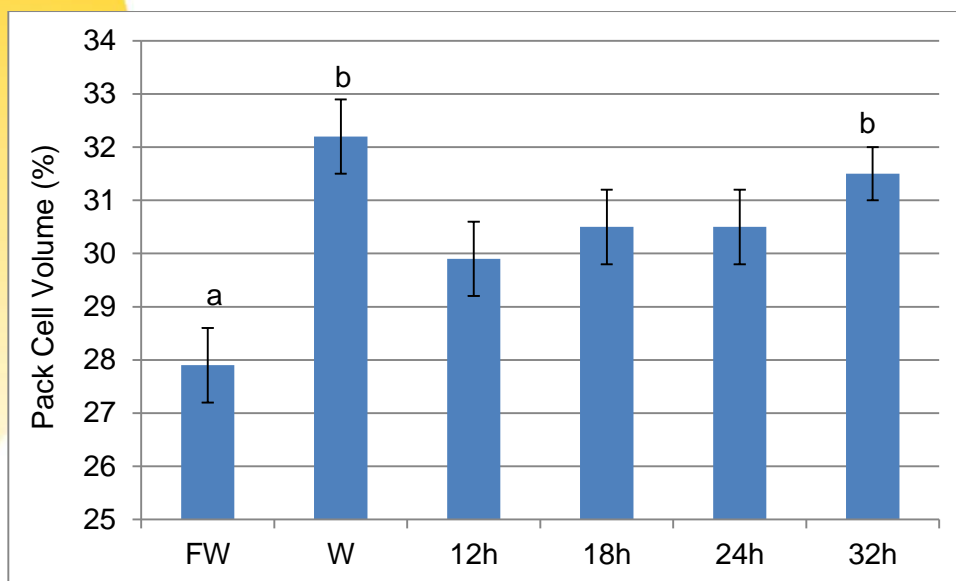
Means with different superscripts (a-b) differ significantly (P < 0.05).

Figure 2-9: Pack cell volume (LS-means ± SEM) by treatment for Replicate 2
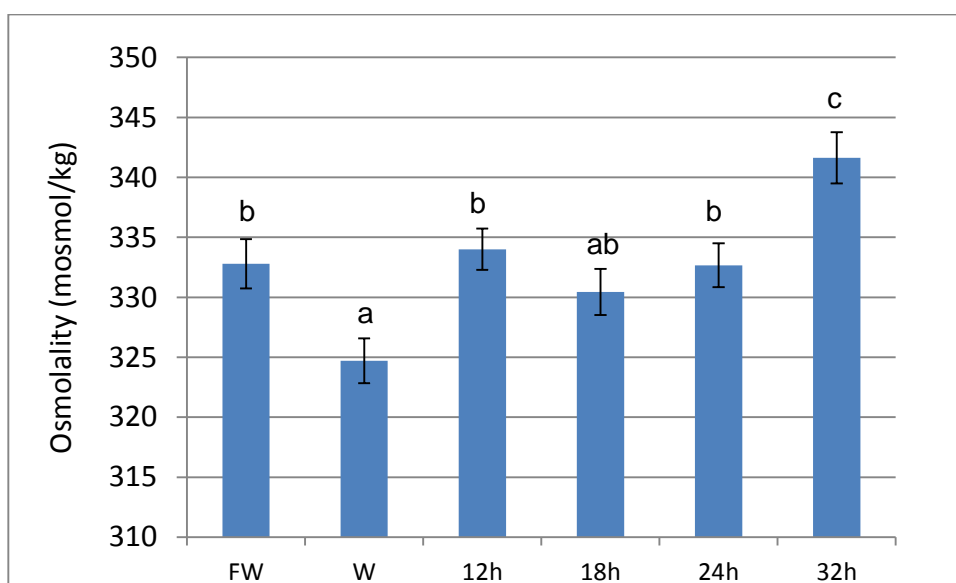
Means with different superscripts (a-c) differ significantly (P < 0.05).

Figure 2-10: Osmolality (LS-means ± SEM) by treatment for Replicate 2

2.3.3 Results summary

Table 2-4: Visual description of the behavioural and physiological results for Experiment 1

	FW	W	12h	18h	24h	32h
Behaviour						
Head out	(grey)	(white)	(grey)	↑ (black)	(grey)	(grey)
Head up	(grey)	(white)	↑ (black)	(white)	↑ (black)	(grey)
Head in feeder	(grey)	(white)	↑ (black)	↑ (black)	(grey)	(grey)
Inactive	(white)	↑ (black)	(white)	↑ (black)	(grey)	(grey)
Not visible	(grey)	↑ (black)	(grey)	(white)	(grey)	(grey)
Physiology						
Weight loss	(white)	↑ (black)	↑ (black)	(grey)	↑ (black)	↑ (black)
Corticosterone	(white)	(white)	↑ (black)	↑ (black)	(grey)	(white)
Packed cell volume	(white)	↑ (black)	(grey)	(grey)	(grey)	↑ (black)
Osmolality	↑ (black)	(white)	↑ (black)	(grey)	↑ (black)	↑ (black)

Squares with different colours (black, white) differ from each other (P < 0.05) except grey which do not differ from black or white (P > 0.05). The arrows indicate the direction of change.
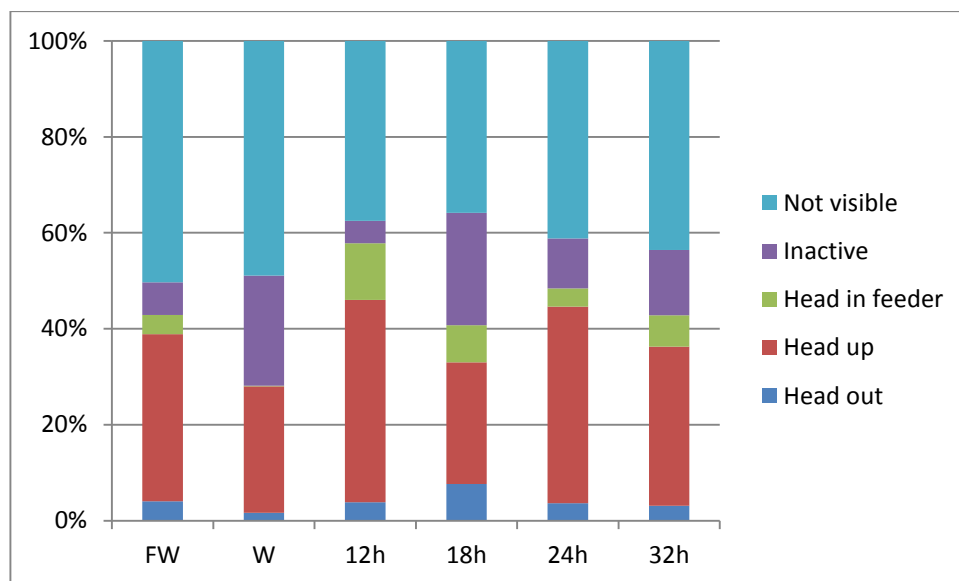
Figure 2-11: Graphical description of the behavioural results for Experiment 1
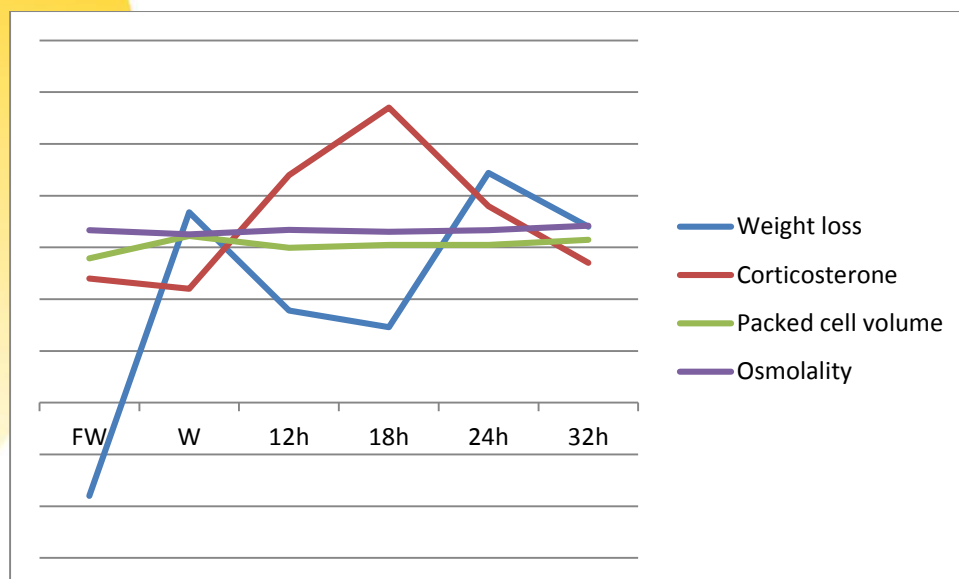
Units are arbitrary for all physiological variables to fit on a common scale.

Figure 2-12: Graphical description of the physiological results for Experiment 1

2.4 Discussion

The previous project (AECL MCCP: 2009-320) identified several physiological changes as time off water and feed increased, such as osmolality, packed cell volume and plasma electrolytes concentration. The aim of this experiment was to investigate behavioural changes that could be used in the interpretation of the welfare implications of water and feed deprivation, given that hens first try to adjust behaviourally in their attempt to cope with a challenging situation. We therefore expected to see an increase in searching behaviour, followed by a higher occurrence of behaviours such as pacing, aggression, or preening, and ultimately reduced activity ('lethargy'). The results showed that behavioural changes occurred at 12h and 18h, suggesting that this is a period during which hens adjusted their behaviour in response to the thwarting situation (**Table 2-4**). Nevertheless, behavioural changes do not necessary equate strictly to a state of compromised welfare, as behaviour is primarily a coping strategy to adapt to change.

The most frequent behaviour observed was head up, occurring in about 25-45% of the scans. More hens in the 12 and 24h treatments had their head up compared to those in the W control treatment, off feed for 32h, or those in the 18h treatment. This difference is difficult to interpret given that the number of hens with their head up increased at different times.

The second most frequent behaviour was inactivity, which corresponded to the hen being immobile and not performing any obvious behaviour. Inactivity was higher for hens in the 18h treatment and the W control treatment, off feed for 32h, compared to hens in the FW control or the 12h treatment. However, inactivity did not simply increase as time off water and feed increased: hens in the 24h and 32h treatments did not show higher levels of inactivity than hens in the FW control treatment. Hence, these results do not support our initial prediction that hens became lethargic past a particular time off feed and water.

The proportion of hens observed with their head in the feeder was higher in the 12h and 18h treatments compared to hens in the W control, which also had no access to feed for the length of the test. This difference in this behaviour suggests that hens were likely displaying an active searching behaviour or a redirected behaviour from 12h onwards, but this difference vanished at 24h and 32h. It also supports the interpretation that hens placing their head in the feeder had more to do with water removal than merely the absence of feed, since the W

control which had ad libitum access to water but no feed were rarely seen with their head in the feeder. Furthermore, hens would normally obtain their water from nipple drinkers located at the back of the cage whereas the feeder was located in front of the cage.

Head out increased for hens in the 18h treatment. The reason for hens to put their head out through the bars is unclear, but could be interpreted either as an attempt to access the feeder, escape or search for resources. Both head out and head in feeder showed a significant interaction of treatment × hour of observation, but the low numbers for these values are probably responsible for statistically significant but biologically negligible changes.

The advantage of our methodological approach, by comparing behaviours for the last 12h at the same time of the day for all treatments, allowed us to control for circadian rhythm for both behavioural and physiological variables. We can reasonably assume that changes before these last 12h will be seen in other treatments with duration exceeding 12h (**Figure 2-1**).

The amount of scans classified as not visible reflects the difficulty of conducting behavioural observations in a conventional cage commercial-like setting. On average, 2 out of the 5 hens in the cage were not visible. It was difficult to get a perception of the depth in the cage, and at the stocking density used ($550cm^2$ in accordance with the Model Code of Practice), 1 hen located at the front of the cage could often block the observer's view of the back of the cage or of other hens. The difficulty to conduct detailed behavioural observations in this commercial-like setting (low visibility and angle of vision) also resulted in the inability to differentiate different types of abnormal behaviours as we initially intended, whether these were redirected behaviours (behaviours directed toward an inappropriate target, for instance pecking at other features of the cage) or displacement behaviours (behaviours displayed out of context, such as excessive feather-preening or aggression). The quantification of the prevalence of these different types of abnormal behaviours in future research would prove useful.

In terms of the physiological changes, the hens did lose weight as a result of feed and water removal, with the largest effect seen at 24h and 32h off water and feed, but also after 32h off feed only. This is consistent with our previous project, which found higher weight loss at 24, 28 and 32h. The similarity between the weight loss for hens in the 32h off water and feed and the hens solely off feed for 32h support that most of the weight loss is due to water rather than feed withdrawal.

Corticosterone is a stress hormone in avian species that is released in response to a perceived stressor. Corticosterone has many actions to ameliorate stress including mobilization of energy, and it is generally accepted that increased concentrations of corticosterone in the plasma indicate that the animal is experiencing a stress response, including water deprivation (Broom and Johnson, 1993). Corticosterone concentration increased for hens in the 12h and 18h treatment, but returned to baseline levels at 32h, both for the 32h off feed and water and the 32h off feed only treatments. This result is different from the previous study, which showed significant elevations in corticosterone concentration from 12h onwards, with still elevated concentrations at 24 and 32h. The reason for this discrepancy in corticosterone concentration changes between the 2 projects is difficult to explain, but is likely affected by the low statistical power due to the use of only 1 replicate in the present study and consequently the low sample size. It could also be due to the social environment, the method of collection or other environmental variables.

The packed cell volume is a measurement of the percentage of blood volume that consists of the red blood cell fraction, with a higher percentage due to a higher red blood cell content and a lower plasma content, indicative of fluid loss and dehydration. Packed cell volume was significantly higher for hens in the 32h off water and feed treatment and the 32h off feed only treatment. This is in agreement with our previous project in which we found higher packed cell volume at 28 and 32h off water and feed.

Osmolality is a measurement of the total number of dissolved particles (in moles) present in a kilogram of water (Chloe and Strange, 2009). Osmolality of chicken plasma provides a sensitive measure of dehydration because it is indicative of the concentration of solutes in the extracellular fluid in the hen's body. In the present experiment, osmolity showed a significant increase between 32h off water and feed compared to the baseline of 32h off feed. Nonetheless, the control treatment with ad libitum access to water and feed had a higher osmolality than the 32h off feed, probably explained by the fact that feeding increases osmolality. Osmolality was the most pertinent finding in relation to hen welfare in the previous AECL project (MCCP: 2009-320), with a 2% loss of body fluid at 12h, and between 6 and 7% thereafter at 24, 28 and 32h. Furthermore, Knowles et al. (1995) reported that the plasma osmolality of laying hens increased with deprivation of both food and water for 24h (+0.03% at 17°C and +1.3% at 23°C) when compared to unrestricted hens. Osmolality has also been reported to increase in broiler chickens following 24h (Arad et al., 1985) and 48h (Zhou et al., 1999) of water deprivation (+10% and +9%, respectively), and by +7% in laying hens after 24h food and water deprivation (Koike et al., 1977). The low statistical power for most of the physiological variables in this project, due to the inability to use 1 of the 2 replicates and consequentyly low sample size, likely explains discrepancies with the findings from the previous AECL project (MCCP: 2009-320). Although the patterns of physiological changes remain similar, the physiological changes from the previous project have superior statistical power and therefore should be used in conjunction with the behavioural changes observed in this study.

The previous project imposed deprivation of water and feed simulatenously, as this is representative of typical transport conditions. However, this project attempted to dissociate the effects of water and feed deprivation from the effects of solely water deprivation by using two control treatments: one with access to water and feed ad libitum and another one with access to water but no access to feed for the 32h duration of the test. The similarities in physiological changes (weight loss, corticosterone concentration, packed cell volume) for hens in the 32h off feed and water or 32h solely off feed support that water deprivation, rather than feed deprivation, had the largest effect. It does go along with previous assumptions that water deprivation is of greatest concern than feed deprivation for the welfare of an animal.

We tested a 'comb colour' scale developed by the Bristol Welfare Assurance Program (Leeb et al., 2005), allegedly to detect potential acute diseases that can cause a cyanotic or violet comb. Our rationale was based on the fact that dehydration and the associated reduction in body fluids increases blood viscosity and reduces blood circulation. Therefore, hypothetically, this reduced blood circulation could lead to changes in comb colour, given that the comb is a highly perfused area in laying hens. We tested this measure as a potentially practical, non-invasive, on-farm assessment tool. Unfortunately, comb colour score did not vary according to treatment. The very low statistical power of the test strongly suggests that a larger sample size is required. It is also possible that this visual scoring system, from 1 to 7, was not sensitive enough to detect differences. Further research could also be conducted using quantitative methods such as a colorimeter, although this is likely less pratical and accessible for on-farm assessment or as a measure to be taken during transport.

In conclusion, behavioural changes occurred as early as within the first 12h (first time point) and 18h, suggesting that this is a period during which hens adjusted their behaviour in response to the thwarting situation. These behavioural changes preceded the physiological changes at 24h and 32h, observed here and in the previous project. Unfortunately, commercial conditions do not lend themselves to video recordings of sufficient quality for a detailed behavioural analysis as we initially intended, to investigate the occurrence of redirected and displacement behaviours such as preening, pacing, aggression toward conspecifics or feather pecking. Experiment 2 was therefore useful as a complimentary approach to assess the motivation of hens to drink, hence the extent to which some of these behavioural changes appear in more controlled conditions.

3 Experiment 2: Effects of time off water on the motivation to access water

3.1 Rationale

Since the demand for water is inelastic in most animals, motivation should be high to work for water. Squeezing through narrow openings has previously been validated in laying hens (Cooper & Appleby, 1996) to assess the level of motivation to access a resource and in turn the importance of the environmental resource. This method also has the advantage of requiring minimal training. We predicted that higher dehydration times should lead to a higher price paid to access water, in this case willingness to squeeze through a narrow opening.

3.2 Methods

3.2.1 Housing

The project was approved by the University of Melbourne Ethics Committee (approval number 1212689.2), in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty, 39 week-old, Hy-line brown laying hens previously housed in conventional cages were obtained from a commercial farm and transported to the Victorian Department of Environment and Primary Industries facilities in Werribee, Victoria. Hens were individually caged (61 × 50 × 45cm) in 1 shed, with visual contact with their neighbours, and allowed 4 weeks to acclimatise to their new environment prior to the start of the tests. They were kept on a 16h light schedule (0500-2100h) and fed ad libitum a formulated layer diet (formulated and mixed by the source farm, 15% crude protein). The temperature ranged between 18 and 24°C with a relative humidity of 50-60%.

3.2.2 Testing apparatus

The testing apparatus was placed in an adjacent shed and consisted of 4 test cages placed at each corner of the shed and visually separated from each other. Each test cage consisted of 2 conventional cages, each identical to the home cage, and connected by a middle door (**Figure 3-1**). This door was adjustable to various widths by sliding the sides of the wooden frame and locking them according to the appropriate width. The hen was placed in the control side whereas the water side was identical except for the presence of a nipple drinker, identical in type and location to the one present in their home cage. A camera was placed in front of the cage and videos were recorded on a computer. The testing cage was divided in 3 zones: the 'control side', the 'water side', and the 'water quarter' (**Figure 3-1**). A feed trough was present in front of the cage and the front side consisted of horizontal bars whereas the 3 other sides were solid-sided. Light intensity, temperature and humidity in the testing cages were kept to similar levels as in the home cages.
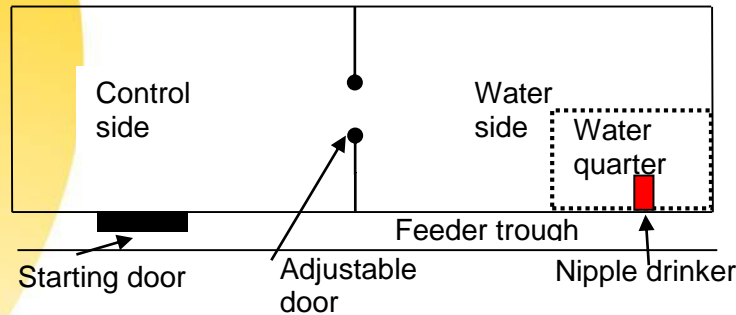
Figure 3-1: Testing apparatus for the motivation test in Experiment 2

3.2.3 Door gaps

The crossing of a narrow vertical door gap is a methodology that has been previously validated to assess the motivation of laying hens to assess a resource on the other side (Cooper & Appleby, 1996). These researchers found that hens with an average width of 117 mm generally can squeeze through a 95 mm-wide vertical gap, with some effort, when highly motivated to access a nest prior to oviposition. In order to adapt that methodology to our hens' phenotype, the width of each hen at the widest point of the shoulders between the external sides of the wings were measured 3 times, 3 days apart, prior to the start of the experiment. Using our average hens' width of $127 \pm 3mm$ (means ± S.D.), we chose 100, 120, 135 and 150 mm-width door gaps, proportionally equivalent to 4 of the 5 door-widths used by Cooper & Appleby (1996).

3.2.4 Time off water treatments

Each hen was individually tested with access to water ad libitum (Control treatment; 0h) or 12, 18, 24 or 32h after water removal. These times were derived from Experiment 1 and a previous AECL experiment (MCCP: 2009-320). Water was removed at various times of day according to the treatment (0200h, 1000h, 1600h, or 2200h) and the hens were tested between 0900 and 1130h the following day in a random order. Feed was still available in the home cage for all treatments but not in the testing cage.

3.2.5 Tests

Each hen was given an acclimatisation period by being placed individually in the testing apparatus for 15min 6 times, twice over 1 week, and finally once 30min prior to the start of the test to explore and learn the location of the nipple drinker, using the largest door gap of 150mm.

In order to minimise the frequency of water removal, the hens were tested using an incomplete randomised block design by subjecting each hen to 10 test sessions out of a total of 20 combinations (5 treatments × 4 door gaps). All hens experienced each treatment twice, but door gaps were randomised across hens. This ensured that each possible combination was tested 10 times. The incomplete randomised block design also controlled for individual variability and sequence of testing effects. A period of 62h was given between each test, and deemed sufficient on the basis of physiological return to baseline (Arad et al., 1985; Koike et al., 1983), allowing for 2 tests per week for a total of 5 weeks. The test started as soon as the experimenter closed the door of the testing cage and lasted for 30min, after which the hens were returned to their home cage with ad libitum access to water.

3.2.6 Data collection

Videos were analysed with the Observer software (version XT 8.0, Noldus, The Netherlands) with a continuous recording method using the ethogram shown in **Table 3-1**. The main measures derived from the ethogram were the latency to attempt passage of the door gap from the control side to the water side, numbers of successful and unsuccessful attempts, latency to reach the nipple drinker for the first successful passage into the water side, location (control side, water side, water quarter), drinking frequency and duration, walk, stand, crouch, peck feeder, peck walls, preen, body shake, wing flap, head poke, head flick and escape attempts. All observations were conducted by a single observer who was blind to treatments and testing days were randomly analysed. The eggs were individually collected and weighted after each testing day around 1400h.

Table 3-1: Ethogram used for Experiment 2

Note: Location and Behaviours were mutually exclusive categories

Location	
Control side	The hen has its two feet in the control side the test cage.
Water side	The hen has its two feet in the water side the test cage.
Water quarter	The hen's feet are a body width from the drinker into the water side.
Behaviours	
Drink[1]	The hen's beak is in contact with the drinker in the water side.
Preen[1]	Straightening of the feathers with the beak.
Walk[1]	The hen is moving around the area of the cage.
Stand[1]	The hen is stationary in the cage and up on its two legs.
Crouch[1]	The hen has its body lower than standing position, in contact with bottom of cage.
Peck at feeder[1]	The hen's beak is in contact with the feeder.
Peck at walls[1]	The hen's beak is in contact with the walls or floor.
Escape attempt[1]	An active attempt to escape from cage. Both neck and feet are on the front barts of the cage during attempt.
Head Poke[1]	Hen pokes head outside of cage door.
Body Shake[2]	Hen's entire body shakes, fluff feathers.
Wing Flap[2]	Hen flaps her wings away from her body.
Head flick[2]	Hen moves head in a short, sharp motion to the side.
Successful Cross[2]	Hen moves through door gap from the control side to the water side.
Unsuccessful Cross[2]	Hen attempts to move through the door gap from the control to the water side but remains in the control side of the cage.
Other[1]	Egg laying, scratching head with feet, bill wiping or any other behaviours not listed above.

[1] *When interruptions in this behaviour of less than 5 sec were observed, the behaviour before and after the interruption was considered to be in the same behavioural bout.*

[2] *These behaviours were only recorded as events due to their short duration.*

3.2.7 Statistical analysis

All data met the criteria for normality and homogeneity of variance. Data were analysed using a mixed model (Proc Mixed, SAS Inst. Inc., Cary, NC, USA), with a model that included day of testing, treatment, door gap and their interaction as fixed effects, hen as a random effect, and accounted for repeated measures over days with hens as subjects. Effects of testing cage location and order of testing within a daily testing session were included in the model when significant. When significant differences ($P < 0.05$) were detected, appropriate Tukey–Kramer adjustments were used for pairwise comparisons between all treatments. Data are presented as LS-means ± SEM.

3.3 Results

3.3.1 Crossing of the door gaps

The number of unsuccessful crossings was affected by the door gap ($P = 0.03$; **Figure 3-2**), with more unsuccessful crossings at the smaller door gap of 100mm compared to the 150mm ($P = 0.04$).

The number of successful crosses was also affected by the door gap ($P < 0.0001$; **Figure 3-3**), with a preference for crossing the 135mm door gap compared to other widths (all $P < 0.001$).

The latency to the first attempt to cross was influenced by the door gap ($P = 0.02$), with hens taking longer to attempt to cross 150mm than 120mm ($148 ± 32$ sec vs. $17 ± 32$ sec, $P = 0.02$), but not different from 135mm or 100mm ($45 ± 32$ sec, $P = 0.11$, and $49 ± 32$ sec, $P = 0.12$, respectively). However, the latency to the first successful cross was not influenced by the door gap ($P = 0.11$). The width of the hens was included as a covariate in the model because it had a significant effect on the latency to the first attempt to cross and the latency to the first successful cross ($P = 0.002$ and $P = 0.005$, respectively).

The number of successful or unsuccessful crossings, or the latencies to the first attempt to cross or the first successful cross, were not influenced by treatment (all $P > 0.05$).
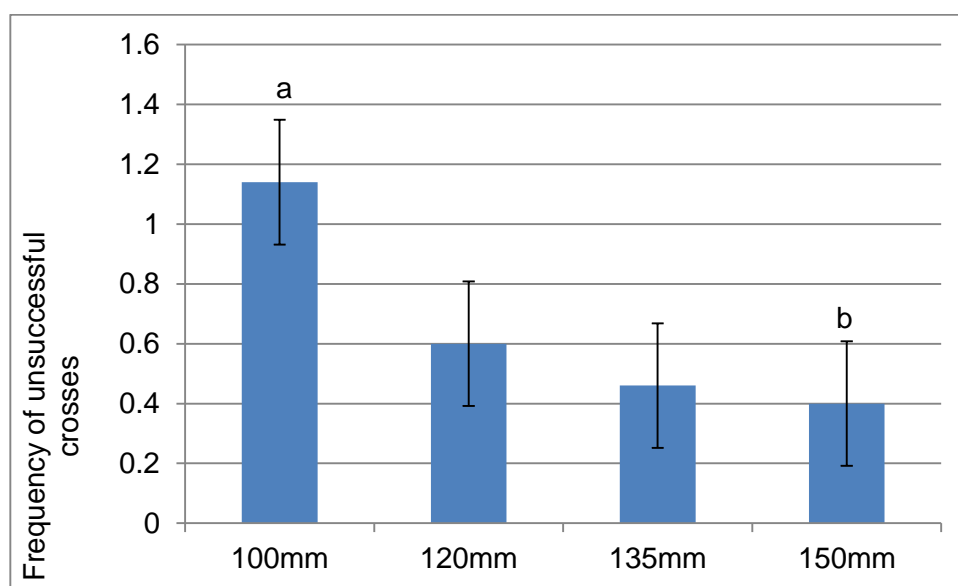
Means with different superscripts (a-b) differ significantly (P <0.05).

Figure 3-2: Frequency of unsuccessful crosses (LS-means ± SEM) by door gaps

Means with different superscripts (a-b) differ significantly (P <0.05).
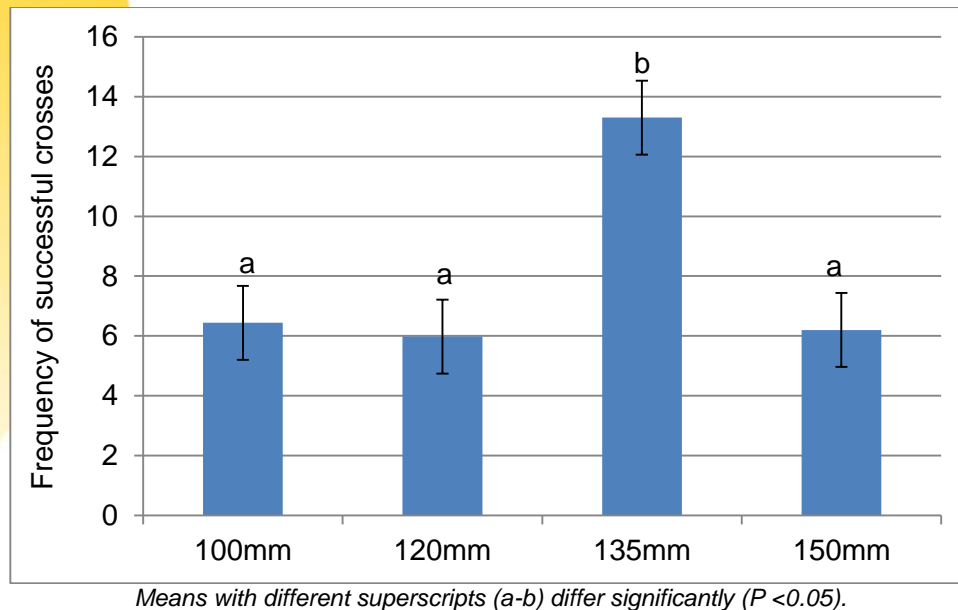
Figure 3-3: Frequency of successful crosses (LS-means ± SEM) by door gaps.

3.3.2 Location

The time hens spent in the control side of the testing apparatus was affected by the door gap (P = 0.03) with the hens spending more time in the control side with the 100mm as compared to the 120mm (734 ± 67 sec vs. 474 ± 67 sec, P =0.03). However, the time spent in the water side or water quarter location were not affected by the door gap (both P = 0.09).

Location was also affected by treatment (all P < 0.01; **Figure 3-4**), with the hens spending more time in the water side for the 32h treatment compared to the 0h treatment (P = 0.008) and more time in the water quarter for 18h, 24h and 32h treatment compared to the 0h and 12h treatment (all P < 0.001 apart from 12h vs. 18h, P = 0.04).

Means with different superscripts (a-b) within a category (Control side, water side or water quarter) differ significantly (P <0.05).

Figure 3-4: Proportion of time spent in the 3 locations of the testing apparatus (mean ± SEM) by Time off water

3.3.3 Drinking behaviour

Duration, frequency or latency of drinking was not affected by the door gaps (all P > 0.1).

The duration of drinking was affected by treatment (P < 0.0001, **Figure 3-5**), with the hens spending more time drinking in the 32h and 24h treatments compared to 18h (P = 0.05 and P = 0.02, respectively), which drank more than in the 12h treatment (P = 0.002), which in turn drank more than 0h (P = 0.01).

The frequency of drinking was also affected by treatment (P = 0.002;

Table 3-2), with hens in the 0h control treatment drinking less frequently than in all other treatments submitted to water removal (all P < 0.04).

Similarly, the latency to drink was also affected by treatment (P = 0.004;

Table 3-2) with hens in the 0h control treatment taking the longest time to reach the drinker than all other treatments (P < 0.001). Latency to drink also showed a day effect (P = 0.0004), with the hens taking longer to drink on the first day compared to all other day (all P < 0.01) apart from day 4 (P = 0.12).

3.3.4 Maintenance and exploratory behaviours

The time spent walking was affected by treatment (P = 0.006;

Table 3-2), with hens in the 24h and 32h treatments walking less than hens in the 0h and 12h treatments (all P < 0.03). Walking was also affected by the door gap (P = 0.04), with the hens walking for longer when the 135mm door gap was in place (459 ± 34 sec) compared to the 100mm or 150mm door gaps (371 ± 30 sec, P = 0.04; 341 ± 28 sec, P = 0.006, respectively) but not different from the 120mm door gap (380 ± 34 sec).

The time spent standing was similarly affected by treatment (P < 0.0001; **Table 3-2**), with the hens in the 32h and 24h treatments standing less than hens in the 12h and 0h treatments (all P < 0.02) and hens in the 18h treatment standing less than hens in the 0h control treatment (P = 0.0004). Standing was not affected by door gap.

Pecking at the empty feeder was also affected by treatment (P = 0.003, **Figure 3-6**), with hens deprived of water for 32h spending more time pecking at the feeder than hens in the 0h, 12h or 18h treatments (P = 0.002, P = 0.03 and P = 0.04, respectively). Pecking at the feeder was not affected by door gap.

Pecking at the walls (overall mean ± SEM: 191 ± 30 sec), escape attempts (overall mean ± SEM: 23 ± 8 sec), crouch (overall mean ± SEM: 30 ± 19 sec), head poke (overall mean ± SEM : 222 ± 23 sec) and the frequency of head flick behaviour (overall mean ± SEM: 3.1 ± 0.4) were not affected by treatment nor door gap (all P > 0.05).
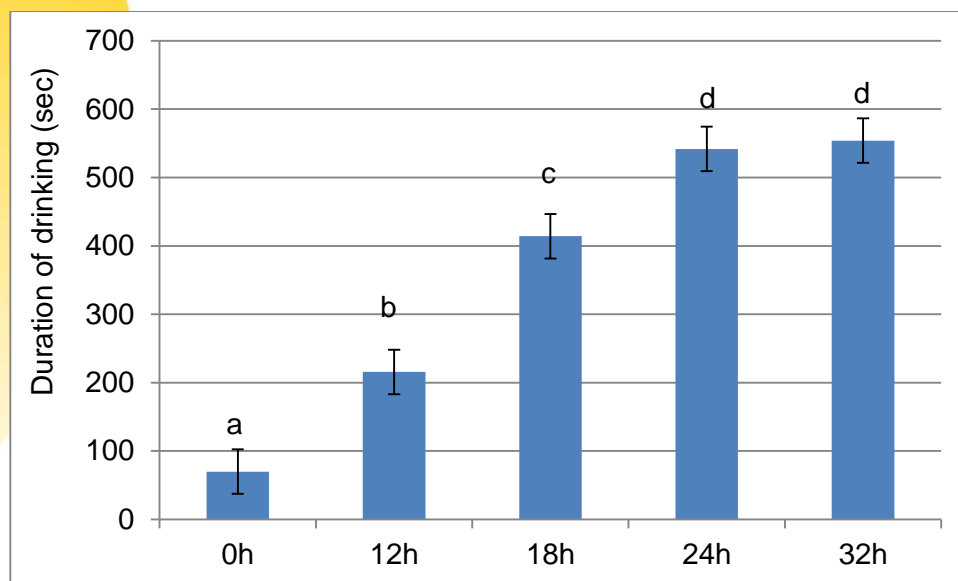
Means with different superscripts (a-d) differ significantly (p <0.05).

Figure 3-5: Duration of Drinking (LS-means ± SEM) by treatment

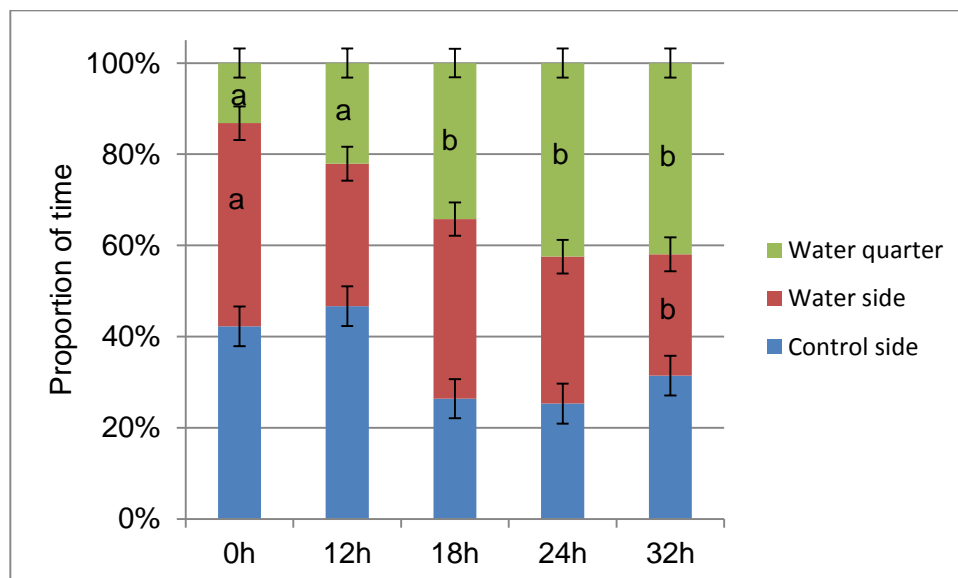**Table 3-2: Behavioural variables affected by treatment (LS-means ± SEM)**

Means with different superscript ($^{a-d}$) differ (p < 0.05).

|	Time off water treatment (h)					P-value
	0	**12**	**18**	**24**	**32**	
Drinking behaviours						
Drink duration (sec)	70 ± 16^a	216 ± 26^b	414 ± 30^c	542 ± 30^d	554 ± 39^d	<0.0001
Drink frequency (number)	1.9 ± 0.2^a	4.4 ± 0.5^b	3.7 ± 0.3^b	4.4 ± 0.4^b	3.7 ± 0.4^b	0.002
Latency to drink (sec)	494 ± 116^a	150 ± 68^b	112 ± 57^b	88 ± 38^b	154 ± 70^b	0.004
Maintenance or exploratory behaviours						
Walk Duration (sec)	450 ± 34^a	458 ± 31^a	372 ± 30^{ab}	346 ± 31^b	313 ± 32^b	0.01
Stand duration (sec)	612 ± 41^a	472 ± 36^{ab}	393 ± 28^{bc}	314 ± 31^c	256 ± 26^c	<0.0001
Peck Feeder Duration (sec)	12 ± 3^a	39 ± 11^a	40 ± 10^a	71 ± 18^{ab}	125 ± 35^b	0.0026
Comfort behaviours						
Preen Frequency (number)	1.2 ± 0.2^a	0.9 ± 0.2^{ab}	0.6 ± 0.2^{ab}	0.4 ± 0.1^b	0.6 ± 0.1^{ab}	0.01
Body Shake Frequency (number)	1.5 ± 0.2^a	1.4 ± 0.2^{ab}	1.0 ± 0.1^{bc}	0.8 ± 0.1^c	1.1 ± 0.1^{abc}	0.02
Wing Flap Frequency (number)	1.5 ± 0.2^a	1.0 ± 0.2^{ab}	0.6 ± 0.1^{bc}	0.2 ± 0.1^c	0.4 ± 0.1^c	<0.0001
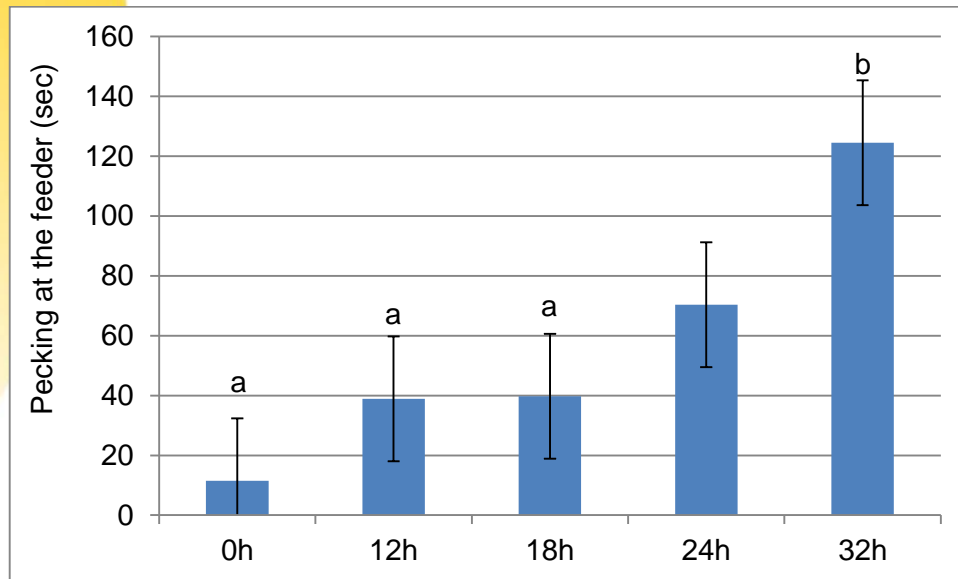
Means with different superscripts (a-d) differ significantly (p <0.05).

Figure 3-6: Duration of Pecking at the feeder (LS-means ± SEM) by treatment

3.3.5 Comfort behaviours

The frequency of preening bouts was affected by treatment (P = 0.01; **Table 3-2**) with less preening bouts in hens at 24h compared to those at 0h (P = 0.01). Door gap had no effect.

Similarly, the frequency of body shake was affected by treatment (P = 0.02; **Table 3-2**), with less body shake in hens at 24h compared to 0h treatments (P = 0.02). Door gap had no effect. Wing flap was also affected by treatment (P <0.0001; **Table 3-2**), with more wing flap seen in hens submitted to 0h control treatment compared to those in the 18h, 24h, or 32h treatments (P = 0.002, P < 0.0001 and P < 0.001, respectively). Door gap had no effect.

3.3.6 Egg weight

Hen-day egg production was 96% in average over the study. The eggs for day 1 were not weighed, however eggs from the remaining 9 days were not affected by treatment or day of testing (both P > 0.1).

3.3.7 Results summary

Table 3-3: Visual description of significant results

Squares with different colours (black, white) differ from each other (P < 0.05) except grey, which do not differ from either black or white (P > 0.05). The arrows indicate the direction for significant changes.

	Time off Water				
	0h	12h	18h	24h	32h
Location					
Control side Duration			↓	↓	(grey)
Water Side Duration		(grey)	(grey)	(grey)	↑
Water Quarter Duration			↑	↑	↑
Drinking Behaviour					
Drinking Duration*		↑	↑	↑	↑
Latency To Drink		↓	↓	↓	↓
Exploratory behaviours					
Walk Duration			(grey)	↓	↓
Stand Duration		(grey)	↓	↓	↓
Peck Feeder Duration					↑
Comfort Behaviour					
Preen Number		(grey)	(grey)	↓	(grey)
Body Shake Number		(grey)	(grey)	↓	(grey)
Wing Flap Number		(grey)	↓	↓	↓
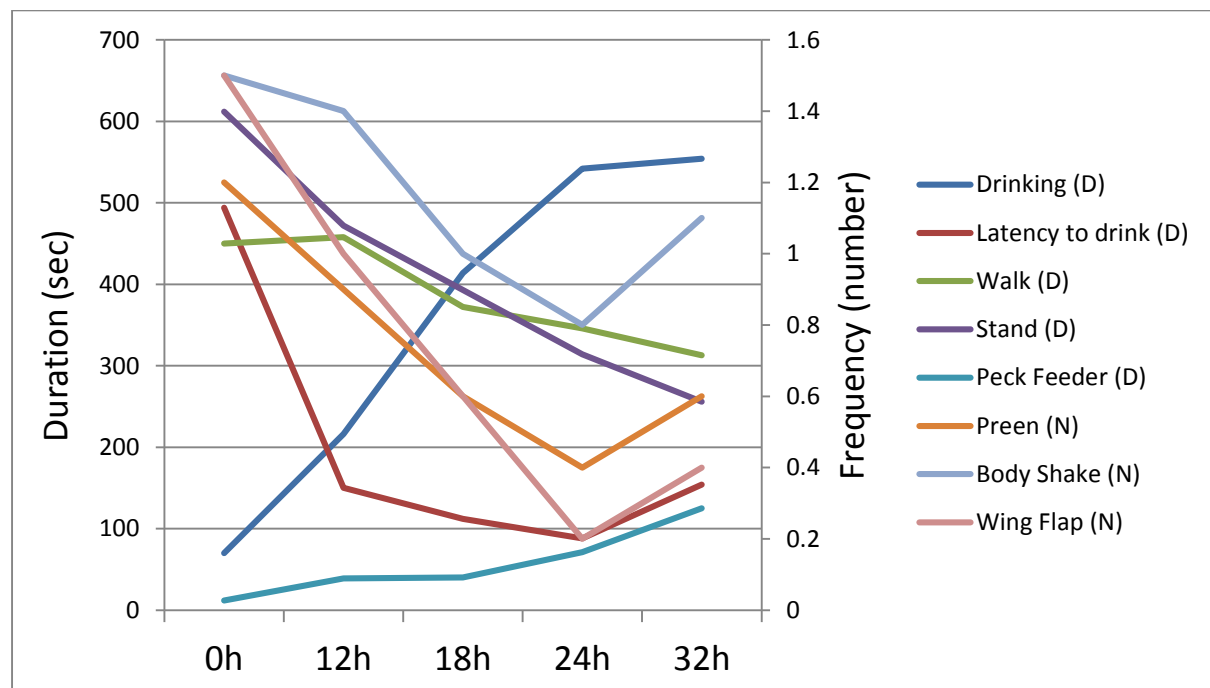
Data presented as duration are followed by (D) and correspond to the left Y-axis scale. Data presented as frequency are followed by (N) and correspond to the right Y-axis scale.

Figure 3-7: Graphical description of significant results.

3.4 Discussion

Overall, the use of narrow vertical door gaps had little effect as a measure of the motivation of the hens to reach the water drinker located in the adjacent side of the testing apparatus. Nonetheless, clear behavioural differences appeared as a result of the length of water removal, with 24h and 32h off water leading to maximum changes on most behavioural variables (e.g. drinking duration), whereas changes were already seen in some behavioural variables at 18h of water removal (e.g. location of the hen close to the drinker, standing).
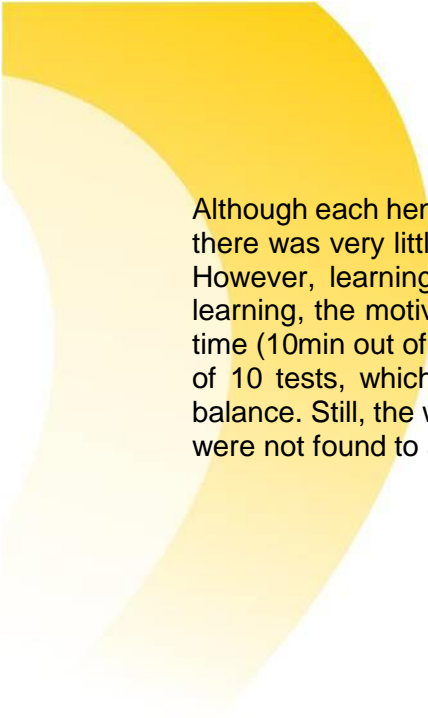
The latency to drink increased in all water removal treatments, indicating that hens were at least moderately motivated to reach the water drinker when water had been removed for 12h or more. As time off water increased, hens drank for longer, but drinking duration reached a maximum at 24h. This is in agreement with findings on broilers (Sprenger et al., 2007), which increased their water consumption according to the length of water removal between 6 and 24h. The fact that drinking duration plateaued between 24h and 32h may be due to the fact that hens are physically restricted to ingest more than this amount of water in such short period of time. The frequency of drinking bouts did not change indicating that the hens adjusted their drinking behaviour by drinking for longer periods of time, rather than more often. The shorter time spent on other behaviours, walking and standing, likely reflected the longer time dedicated to drinking. Hens in the 32h treatment also spent a considerable amount of time pecking at the empty feeder, suggesting that they were highly motivated to the point where they started pecking the feeder, not just the drinker. Pecking at the walls and floor did not change, which support that pecking at the feeder for the 32h treatment was somehow related to the search for water, and maybe to a longer time to reach water satiety and restore homeostasis for these birds upon rehydration (Toates, 1979; Sprenger et al., 2007). It is also possible that the motivations to drink and eat are neurobiologically linked in laying hens, or are due to conditioning: both deprivation of food or water result in an increase in frustration-induced aggression (Haskell et al., 2000). Hence, very high motivation to drink after 32h off water may stimulate both the motivation to peck at the drinker to obtain water and simultaneously to peck at the empty feeder, despite the fact that hens were never feed deprived before the test. In terms of development of the behavioural changes, both the location of the hens close to the drinker in the 'water quarter' and drinking behaviour linearly increased as time off water increased, reaching a plateau at 24h with no differences between 24h and 32h on most behaviours. However, 18h was somewhat intermediate across most behavioural variables and consequently behavioural changes started earlier than 24h and 32h after water removal (**Table 3-3**).

Comfort behaviours were displayed less often as time off water increased, with less preening body shake at 24h, and less wing flap at 18h, 24h and 32h compared to the 0h control treatment. Nonetheless, these behaviours were overall relatively rare. Interestingly, the decrease in the number of preening bouts corresponds to the veterinary empirical knowledge that removing water for 24h decreases feather pecking and cannibalism in commercial flocks (P. Scott, personal communication).

The narrow gap of 100mm hampered movement between the two sides of the testing apparatus, with more unsuccessful crossing attempts. As a result, hens spent more time in the control side where they started the test. Although door gap width had an effect on the hens' crossing from one side to the other, the interaction between door gap and treatment was never significant, which implies that the door gap was more of a physical difficulty irrespective of the motivation to drink. The influence of the hen width on the latency to cross, although expected, does support the interpretation that crossing a narrow door gap represented a physical challenge for the hen, in accordance with Cooper & Appleby (1996). The preference for crossing the 135mm door gap, and the resulting higher locomotion, is an anecdotal but interesting effect. This was not observed by Copper & Appleby (1996). It may be that a slight ruffling or scratching of their feathers had a rewarding effect, since the hens were 127mm wide on average, hence just 8mm less than the 135mm door gap.

Although each hen was submitted to the test 10 times, with different combinations each time, there was very little day effect apart from the longer latency to drink on the first day of test. However, learning occurred quickly as all other days were comparable. Notwithstanding learning, the motivation to reach the water drinker and consequently drink for an extended time (10min out of the 30min test for the 24h and 32h treatment) persisted across the series of 10 tests, which highlight the priority of drinking for hens in a state of negative water balance. Still, the water removal treatments or the cumulative effects from the water removal were not found to affect egg weight or egg production.

4 Implications for industry practices

The present experiment aimed to equate physiological changes induced by time off water with behavioural changes in order to understand its welfare implications for spent hens during transport. A previous AECL project (MCCP: 2009-320) provided physiological evidence that the welfare of spent hens is challenged by deprivation of food and water for 24h and more, using the time points of 12, 24, 28 and 32h. The present project attempted to go further by looking at behavioural evidence in addition to physiological evidence, including 18h as a time point instead of 28h, and adding a control treatment given ad libitum access to water but no feed for 32h.

The results showed that, under favourable handling, social and climatic conditions, the welfare of spent hens became challenged due to water deprivation. Hens changed their behaviour as early as 12h after water deprivation (first time point). Nevertheless, behavioural changes do not necessary equate strictly to a state of compromised welfare, as behaviour is primarily a coping strategy to adapt to change. Physiological changes occurred by 24h, to a similar level to what was seen at 32h, which suggests that a plateau was reached in terms of acute physiological adaptation. Consequently, the results presented in this report, in accordance with our previous report (MCCP: 2009-320), questions the welfare of hens that have water withdrawn for 24h or longer. Nevertheless, there are no clearly defined thresholds indicative of acceptable and unacceptable welfare in the measured responses. When relying on behavioral, physiological, and fitness measures to determine welfare risks, a judgment is made about what degree of change in these indicators is likely to indicate compromised animal welfare. If one favours a conservative decision, the behavioural changes suggested that welfare starts being compromised earlier than 24h after water removal, and probably somewhere between 18h and 24h. However, if one favours the physiological changes, physiological adaptation reached a plateau at 24h, suggesting that 24h appear as the maximum acceptable time off water and that 32h is too long.

In comparison to the previous project, hens were not moved from their cages or handled at any time during treatments. Hence, the behavioural and physiological changes occurred purely as a result of water and feed deprivation. Furthermore, the use of a control treatment in Experiment 1 provided with water ad libitum but no feed for 32h showed that water deprivation, rather than feed deprivation, is responsible for the behavioural and physiological changes observed.

These experiments have been conducted under favourable handling and climatic conditions. It should be recognized that factors other than feed and water deprivation are likely to influence hen welfare during transport, such as the health status of the hens prior to loading, their body condition, stress of handling, social stress of mixing, duration of transport and the weather during transport and lairage. Further research is required to determine what factors specifically influence the welfare of spent hens during transport.

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6 Plain English Summary

Project Title:	The effects of time off feed and water on the welfare of spent laying hens. Phase 2: Behavioural indicators
AECL Project No	1UM122
Researchers Involved	J-L. Rault, P. Hemsworth, A. Tilbrook and P. Scott
Organisations Involved	University of Melbourne, Parkville, VIC; South Australian Research & Development Institute, Roseworthy, SA; Scolexia, Attwood, VIC
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Objectives	This project aims to equate physiological changes induced by water deprivation with behavioural changes in order to understand its welfare implications for the transport of spent laying hens.
Background	The acceptable time length that laying hens can spend without water before welfare is compromised is unknown. In a previous project, physiological measures of dehydration increased with time. However, no scientific literature exists on what can be considered acceptable changes in terms of hen welfare. Hens should first try to adjust behaviourally to water deprivation by showing an increased motivation to access water resources. Hence, behavioural changes should provide useful information regarding the perceived need by the hen to drink.
Research	Experiment 1 investigated the behavioural changes occurring at 12, 18, 24 or 32h after water and feed removal, or solely after 32h off feed, in in conjunction with the physiological measures of corticosterone concentration, packed cell volume, osmolallity, comb colour score, and weight loss. Experiment 2 employed a motivation test using the rationale that higher dehydration times (0, 12, 18, 24 or 32h) should lead to a higher price paid to access water, in this case willingness to squeeze through a narrow opening (150, 135, 120 or 100mm).
Outcomes	Behavioural changes occurred as early as 12h and 18h, preceeding the physiological changes (weight loss, packed cell volume, osmolality) at 24h and 32h. Behavioural differences reaching a plateau at 24h with no differences between 24h and 32h on most behaviours, but some behavioural changes were already apparent at 18h.
Implications	Hens changed their behaviour as early as 12h after water deprivation (first time point). Physiological changes were maximal by 24h, to a similar level to what was seen at 32h, suggesting that a plateau was reached. Consequently, the results presented in this report, in accordance with our previous report (MCCP: 2009-320), questions the welfare of hens that have water withdrew for 24h or longer. The threshold indicative of acceptable welfare remains debatable depending on value-based judgements.
Key Words	Water, Dehydration, Transport, Spent hens, Welfare
Publications	No publications has yet originated from this work

7 Appendix 1. Behaviours by Time off water and feed (LS-means ± SEM) for Experiment 1 - Replicate 1

Significant P-values are highlighted in bold.

	FW (0h)	W (32h F)	12h	18h	24h	32h	Treatment P-value	Hour P-value	Treatment x Hour P-valye	Previous P-value	Treatment x Previous treatment P-value
Head out (%)	5.6 ± 0.8	5.4 ± 0.9	7.3 ± 0.9	7.7 ± 0.8	6.6 ± 0.9	5.7 ± 0.8	**0.002**	**<0.0001**	0.53	**0.05**	**<0.0001**
Head up (%)	43.4 ± 4.1	49.8 ± 4.5	47.9 ± 4.5	45.2 ± 4.0	43.0 ± 4.5	44.2 ± 4.0	0.53	**<0.0001**	0.85	0.30	0.26
Head in feeder (%)	9.9 ± 1.4	4.3 ± 1.6	1.4 ± 1.6	2.2 ± 1.4	1.5 ± 1.5	1.5 ± 1.4	**<0.0001**	**<0.0001**	**0.001**	0.46	**0.0009**
Inactive (%)	4.9 ± 1.3	6.3 ± 1.5	3.8 ± 1.5	5.1 ± 1.3	3.1 ± 1.5	6.3 ± 1.3	**0.05**	**<0.0001**	0.51	**0.02**	0.0008
Not visible (%)	36.0 ± 5.0	34.2 ± 5.6	39.8 ± 5.6	39.8 ± 5.0	45.8 ± 5.6	42.4 ± 5.0	0.22	**<0.0001**	0.90	0.07	**0.002**

8 Appendix 2. Physiology, weight and comb colour score by Time off water and feed (LS-means ± SEM) for Experiment 1 - Replicate 1.

Significant P-values are highlighted in bold.

	FW (0h)	W (32h F)	12h	18h	24h	32h	Treatment x Hour P-valye	Previous P-value	Treatment x Previous treatment P-value
Cage average weight at arrival (g)	2164 ± 61	2088 ± 68	2123 ± 68	2140 ± 61	2178 ± 68	2214 ± 61	0.23	0.24	0.11
Live Weight (g)	2208 ± 46	1976 ± 51	2066 ± 52	2027 ± 46	2086 ± 51	1961 ± 46	**0.03**	0.34	0.06
Weight loss (g)	-44 ± 42	111 ± 47	56 ± 47	114 ± 42	92 ± 47	254 ± 42	**0.02**	0.28	0.96
Corticosterone (ng/mL) *	3.5 ± 0.5	2.1 ± 0.6	4.6 ± 0.6	3.3 ± 0.5	3.2 ± 0.6	3.7 ± 0.5	**0.05**	0.21	**0.003**
Packed cell volume (%)	30.1 ± 0.6	32.8 ± 0.6	30.9 ± 0.6	30.9 ± 0.6	32.3 ± 0.6	33.2 ± 0.6	**0.005**	0.35	0.28
Osmolality (mosmol/kg)	335 ± 2	321 ± 3	333 ± 2	330 ± 2	335 ± 2	341 ± 2	**<0.0001**	0.06	**0.004**
Comb colour score	4.0 ± 0.3	5.0 ± 0.3	4.8 ± 0.3	4.85 ± 0.3	4.8 ± 0.3	4.9 ± 0.3	0.11	**0.05**	**0.001**

Corticosterone concentration is presented as untransformed means but was analysed using a log transformation

9 Appendix 3. Behaviours by hour of observation for each treatment (LS-means ± SEM) for Experiment 1 - Replicate 2

Head out behaviour overtime

Head up behaviour overtime

Head in feeder behaviour overtime

Inactive behaviour overtime

Legend: 12h, 18h, 24h, 32h, FW, W

Y-axis: % of scan observations

X-axis: 0430h, 0530h, 0630h, 0730h, 0830h, 0930h, 1030h, 1130h, 1230h, 13h30

Not visible behaviour overtime

Legend: 12h, 18h, 24h, 32h, FW, W

Y-axis: % of scan observations

X-axis: 0430h, 0530h, 0630h, 0730h, 0830h, 0930h, 1030h, 1130h, 1230h, 13h30