The Impact of Excreta/Excreta Gas Control Strategies on the Behaviour and Physiology of Laying Hens

by

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ABSTRACT

THE IMPACT OF EXCRETA/EXCRETA GAS CONTROL STRATEGIES ON THE BEHAVIOUR AND PHYSIOLOGY OF LAYING HENS

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Commercially housed laying hens are exposed to excreta/excreta gases that would not arise in their natural habitats. Limited information is available on how exposure to excreta, excreta gas, or their control strategies (fresh litter, litter amendments, and low-protein diets) impact laying hens. Therefore, this study was designed to understand the behavioural and physiological implications of exposure to excreta/excreta gases on laying hens. Four experiments comprise this thesis. The first experiment tested hens' behavioural response to air/excreta gas mixture in a chamber and found that hens prefer fresh air over excreta gas (Chapter 3). The second experiment then assessed hens' relative preference for using non-soiled or soiled scratch pads in enriched cages and reported more foraging in excreta-soiled compared to non-soiled pads (Chapter 4). In a consumer-demand setup, the third experiment assessed hens' motivation to access unsoiled litter, soiled litter, soiled litter treated with an acidifier, or no litter substrate in floor pens. The outcome showed that hens possessed a relative preference for litter substrates over no litter but displayed no preference for litter (soiled/unsoiled) type (Chapter 5). Finally, the last experiment found that nitrogen-reduced diets did not impact

physiology or learning ability of laying hens taught a discrimination reversal-learning task (Chapter 6). These results provide the first glimpse into how excreta and excreta-gas environments impact laying hen welfare.

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPP	Average maximum price paid
ANOVA	Analysis of Variance
AST	Aspartate aminotransferase
AUP	Animal user protocol
avP	Available phosphorus
A_w	Water activity
BBB	Blood-brain barrier
$C_5H_4O_3N_4$	Uric acid
Ca	Calcium
CCAC	Canadian Council of Animal Care
CH_4	Methane
CO	Carbon monoxide
$CO(NH_2)_2$	Urea
CO_2	Carbon dioxide
CP	Crude protein
CPK	Creatine Phosphokinase

EFC	Egg Farmers of Canada
EFSA	European Food Safety Authority
FLHS	Fatty liver hemorrhagic syndrome
GGT	Gamma-glutamyl transferase
GLIMMIX	Generalized linear mixed model
H_2O	Water
H_2S	Hydrogen sulphide
IU	International unit
LPER	Low-protein energy-rich
ME	Metabolizable energy
N_2	Nitrogen
N_2O	Nitrous oxide
Na	Sodium
NFACC	National Farm Animal Care Council
NH_3	Ammonia
pH	Potential hydrogen
PIT	Passive integrated transponder
PLT®	Poultry Litter Treatment®
PROC	Procedure

RFID	Radio frequency identification technology
SAS	Statistical Analysis System
SEM	Standard error of the mean
U/L	Units per litre
wk	Week
WAP	World Animal Protection »

1 General Introduction

In Canada, approximately 25 million laying hens are raised for egg production with Ontario alone contributing to over 35% of the production (EFC, 2018). Approximately 77% of Canadian laying hens are housed in conventional cages (EFC, 2017) and the remaining hen population is kept in alternative housing systems such as enriched cages, litter-based barns, and free-range systems. Barns and free-range systems vary widely in design and management, mainly governed by the requirements and available space. Barn systems can be single-level or multi-tier structures. Single-level barn systems may be litter-based systems that give hens the opportunity to access litter substrates throughout the production period or have perforated flooring (slatted floors) (Elson, 2004; Shields and Duncan, 2009). Multi-tier barn systems (referred to as aviaries), designed to use vertical space, allow hens to navigate in complex three-dimensional space (Kozak et al., 2016). Compared to conventional battery cages, alternative housing systems allow hens to express their natural behaviours such as nesting, foraging (pecking, scratching and searching for feed), perching, and dustbathing (Shields and Duncan, 2009).

Different housing systems are designed in different ways to manage laying hens' excreta. Conventional and enriched cages are furnished with manure belts underneath to collect excreta (Elson, 2004). In single-level barn systems, hens are provided with loose litter substrates on the floor and such systems may be deep-litter systems or designed with perforated flooring allowing manure to drop below into a pit (Shields and Duncan, 2009). Multi-tier systems have both floors with the provision of litter substrates and manure belts built under the perforated or wire platforms to allow excreta to be removed (Elson, 2004).

Poultry housing systems containing manure belts allow for the removal of excreta in regular intervals to avoid excreta build-up whereas, in deep-litter systems, litter substrates containing excreta are usually removed at the end of the production cycle causing a build-up litter over the production period.

Although most hens are kept in conventional cages in Canada, alternative housing systems are becoming increasingly popular following the pressure from public, advocacy groups such as Humane Society International, and retails chains such as McDonald's (McDonalds, 2015), Tim Hortons (Tim Hortons, 2012) and Burger King (WAP, 2017). In February 2016, Egg Farmers of Canada announced an industry-wide transition away from conventional cage housing systems to alternative systems (EFC, 2016) in response to consumer preferences and scientific studies addressing the welfare of laying hens.

Alternative housing systems undoubtedly provide laying hens with resources important to their welfare (EFSA, 2005). However, these housing systems can give rise to other health and welfare concerns because of poor air and litter quality if not managed properly (Rodenburg et al., 2008; David et al., 2015). Housing systems with the provision for litter substrates may lead to the production of airborne dust and ammonia (NH_3) (Gholap, 2012). Air and litter quality have already been identified as research priorities by the Canadian committee of scientists responsible for writing Canadian Codes of Practice for the care and handling of chickens (Poultry Code of Practice Scientific Committee, 2013).

This thesis revolves around the knowledge gap mainly related to the health and behavioural implications and hens' preference for hygienic versus excreta/litter-polluted environments.

With the transition to the litter-based[1] alternative housing system, it is essential to identify whether hens prefer to live in litter-based housing systems and to identify the behavioural impacts of living in litter-based housing systems with poor air quality.

[1] In this thesis, litter-based housing system will refer to the laying hen housing systems, where there is provision of litter substrates, whether it is single tier or multi-tier barn system.

2 Literature Review

2.1 Introduction

This literature review is divided into four sections to review the published information surrounding excreta-soiled environments in laying hen houses, its impact on laying hens, and excreta and ammonia control strategies. The literature presented and gaps identified will serve to provide the basis for Chapters 3, 4, 5 and 6. The first section **Litter** (2.2, 2.3, and 2.4) explores litter environments in laying hen houses, exposure of laying hens to litter, excreta as well as to NH_3 and their impacts on laying hens. The second section **Excreta and ammonia control strategies** (2.4) will provide an overview of the strategies used to clean soiled litter substrates to control NH_3 production in laying hen houses with a focus on chemical litter amendments such as Poultry Litter Treatment (PLT®) and dietary approach (such as reduced protein diet). The third section **Key highlights of the literature review** (2.5) will point out key findings from the literature review. The final section **Gaps in the literature** (2.6) will review the gaps from the studies mentioned in this Literature review to provide the rationale behind Chapters 3, 4, 5 and 6.

2.2 Litter

2.2.1 Poultry litter

Poultry litter is a combination of bedding material (shavings, rice hulls, etc.), poultry excreta[2], dander, feather, spilled feed, water and other components (Terzich et al., 2000; Sistani et al., 2003). Canadian Code of Practice for the Care and Handling of Pullets and Laying Hens

[2] In this thesis, the term excreta is used to represent droppings or manure produced by laying hens. Excreta should not be confused with poultry litter.

defines poultry litter as “the combination of bedding and/or bird excreta, feathers, feed, dust, and other materials on floors of bird housing systems” (NFACC, 2017). Poultry litter serves as a cushioning material for birds while also providing a layer of insulation above the ground (Hinkle, 2010). Poultry litter also binds moisture and provides a non-slippery surface for birds to walk on (Garcês et al., 2013). In addition, birds use litter as foraging, nesting and dustbathing material (Cooper and Albentosa, 2003; Moesta et al., 2008; Campbell et al., 2016; Campbell et al., 2017). Good litter management is crucial for bird’s health and welfare, as well as that of stock people working in poultry houses (Ritz et al., 2004; Hinkle, 2010).

Good litter management practices inside the farm require keeping litter dry (Lister, 2009), avoiding caking (Miles et al., 2011; Dunlop, 2017) and removal of bedding materials or litter substrates at the end of every production cycle (Lister, 2009; Gholap, 2012) to prevent the production of NH_3 and other litter gases. Van Staaveren et al. (2018) reported that approximately 67% of Canadian producers managing litter-based housing systems do not replace litter during the production cycle. Over time, litter becomes crusted in the areas where moisture is excessively high, leading to the formation of cake (Dunlop, 2017). Additionally, litter friability gets reduced decreasing the ability of fresh excreta to be incorporated into the litter, which causes the formation of an excreta layer on the litter (excreta build-up on the litter) (Bernhart and Fasina, 2009; Dunlop et al., 2016). This can contribute to the elevated NH_3 release from the poultry litter. Such litter also gets seeded with pathogens increasing the risks of respiratory diseases (Dunlop et al., 2016). Direct contact with such litter can also lead to painful contact dermatitis (Wang et al., 1998).

2.2.2 Production and composition of excreta in laying hen houses

Poultry excreta refers to the droppings produced by chickens. A laying hen produces approximately 800 g of excreta per week (Han et al., 2018). To provide a context, a commercial poultry production unit consisting of 20,000 hens has an average production of 2,300 kg of excreta each day. Out of total nitrogen, poultry excreta contains 60-65% of uric acid, 10% of NH_3 salts, 2-3% of urea and remains of creatinine (Groot Koerkamp, 1994). Fresh excreta contain about 30% crude protein, mostly derived from uric acid on a dry matter basis (Li and Zhang, 2009; Wang 2013 as cited in Han et al., 2018). As nitrogen content in poultry excreta varies from 13-17 g/kg (Groot Koerkamp, 1994), it can be assumed that there is a potential of a significant amount of NH_3 production inside the poultry barn, especially in houses with high stocking density. Although volatilization of NH_3 (conversion of ammonium to NH_3) is dependent on various factors including pH, temperature, microbiological activities, building type, and excreta treatment method (Arogo et al., 2002), NH_3 solely originates from poultry excreta. Apart from NH_3, laying hen excreta is also a source of other gases, albeit to a lesser degree, such as hydrogen sulfide (H_2S) and methane (CH_4) (Fournel et al., 2012; Brouček and Čermák, 2015).

2.3 Excreta exposure and foraging in laying hens

Foraging, in laying hens, involves pecking, scratching and locomotor activities usually accompanying feeding behaviour (Lindqvist, 2008). Laying hens are highly motivated to forage (Bubier, 1996; Lindqvist et al., 2002). The ancestors of domestic hens, Red Junglefowl, spend approximately 60% of their time foraging (Dawkins, 1989). Naturally, laying hens forage to select mixed and diverse diets based on their need (Weeks and Nicol,

2006). Lack of foraging opportunity may result in redirected foraging behaviour; in some cases, even feather pecking in laying hens (Huber-Eicher and Wechsler, 1998).

In natural environments, laying hens have ample opportunities to perform foraging behaviour. However, modern laying hen houses[3] have very limited foraging opportunities. For example, conventional cages do not have any specific foraging area for laying hens. In case of enriched cages, there may be some provision of floor area in the form of a scratch pad to provide foraging area (Appleby et al., 2002; Lay et al., 2011). The scratch pads/plastic mats usually get soiled with excreta throughout the production cycle (Jones et al., 2015; personal observation), causing hens to forage on excreta and occasional consumption of excreta (personal observation). Soiling of scratch pads with excreta leads to poor hygiene, which can become a health and welfare concern for laying hens (EFSA, 2005). In addition, farmers either remove or clean excreta-soiled scratch pads less frequently during the production cycle (Guinebretière et al., 2012). Occasional cleaning of scratch pads can lead to the accumulation of considerable amount of excreta.

Further, excreta accumulation can lead to increased production of dust/particulate matter, and NH_3 while also acting as a source of microorganisms such as fungi, viruses, bacteria, and toxins (Turnbull and Snoeyenbos, 1973; Weaver and Meijerhof, 1991; Himathongkham and Riemann, 1999; Zarrin et al., 2010; Imran and Ali, 2014). Non-cage systems usually have loose litter substrates to support the foraging behaviour of hens. However, Van Staaveren et al. (2018) found that over 20% of the non-cage housing systems in Canada

[3] Modern laying hen houses referes to conventional cages, enriched cages, and barns.

had no litter substrates for laying hens. Even if the litter substrates were provided, they were not replaced during the production cycle (van Staaveren et al., 2018). Such litter substrates get soiled with excreta over time, and hens get exposed to excreta and excreta gases. Laying hens are exposed to excreta through different routes: oral (Waldburg-Zeil et al., 2018), dermal (Kaukonen et al., 2016), and inhalation (Nunes et al., 2016).

Studies have reported that many domestic animals forage away from excreta, which is beneficial in reducing parasitic load and diseases (sheep: Forbes and Hodgson, 1985; Cooper et al., 2000; cattle: Michel, 1955; Forbes and Hodgson, 1985). Whereas, a study conducted in mice (Walsh et al., 2013) reported the absence of fecal avoidance while foraging and feeding in contrast to other laboratory mice study reporting selective foraging (Kavaliers et al., 1997). Studies regarding selective foraging and feeding in laying hens are rare. von Waldburg-Zeil et al. (2018) in their study on the preference of laying hens for foraging on diets mixed with excreta reported hens consuming diets mixed with excreta. During the circumstances when other options for foraging are not available, hens prefer excreta as a litter substrate over no litter substrates, increasing the chance of oral and dermal exposure to excreta (Van Staaveren et al., 2018). As there is a significant excreta build-up on the litter bed and scratch pads over time (Groot Koerkamp, 1994), it can be assumed that hens will be foraging and feeding on litter or scratch pads soiled with excreta. However, whether hens prefer or avoid such soiled litter/scratch pads has not been studied.

2.4 Poultry litter as a source of NH_3 and other gases

Poultry litter is a major source of NH_3 (Wheeler et al., 2003; Hinkle, 2010), which is a major noxious gas (Gholap, 2012) in poultry houses. The concentration of NH_3 may vary from farm

to farm depending on the management practices such as stocking density, litter management, ventilation, and diet (Zhao et al., 2015; David et al., 2015). A review by David et al. (2015) reported NH_3 concentrations in poultry houses ranging from as low as 0.4 ppm to as high as 80 ppm. Although concentrations above 20-25 ppm are not recommended (Kristensen and Wathes, 2000; NFACC, 2017), NH_3 concentrations above 25 ppm can occur in commercial poultry facilities (Choinière and Munroe, 1997; Liang et al., 2005; Nimmermark et al., 2009).

Ammonia, a colorless water-soluble and volatile gas, is the result of microbial decomposition of nitrogenous compounds in the poultry excreta, mainly uric acid (Groot Koerkamp et al., 1998). Other compounds such as undigested proteins and urea present in poultry excreta also contribute to NH_3 production; however, to a lesser extent (Groot Koerkamp et al., 1998). Volatilization of NH_3 from uric acid is caused by the uricase enzyme produced by bacteria named *Bacillus pasteurii* (Schefferle, 1965; David et al., 2015), which is found in the poultry litter. Presence of moisture, high temperature, and excreta pH above 7 favors the activity of uricase enzyme favoring NH_3 production in the litter (Senyondo, 2013). Ammonia is generated from poultry excreta through the following reactions (Groot Koerkamp et al., 1998):

$C_5H_4O_3N_4$ (Uric acid) + $1.5O_2$ + $4H_2O \longrightarrow 5CO_2 + 4NH_3$ …….. aerobic decomposition

$CO(NH_2)_2$ (Urea) + $H_2O \longrightarrow CO_2 + 2NH_3$…….....…… urea hydrolysis

Undigested proteins $\longrightarrow$ NH_3…….....…….mineralization

The concentration of NH_3 inside poultry houses can vary in different housing systems. Litter-based housing systems usually are found to have higher NH_3 concentrations compared to conventional or enriched cage systems (David et al., 2015; Shepherd et al., 2015). However, the concentration of NH_3 is dependent on the frequency of litter and/or excreta removal irrespective of the housing system as excreta is the source of NH_3 generation within poultry houses (Nicholson et al., 2004; Liang et al., 2005).

Apart from NH_3, poultry litter is also a source of other gaseous pollutants such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and hydrogen sulphide (H_2S) (Kocaman et al., 2005; Liang et al., 2005; Xin et al., 2011; Prodanov et al., 2016). However, the contribution of gases other than NH_3 is minimal as an aerial pollutant in the poultry houses. Prodanov et al. (2016) conducted a study in 10 laying hen houses, which found an average concentration of CO_2 ranging in between 696.2 and 1466.56 ppm with the standard deviation of 321.39 ppm, while other gases such as carbon monoxide (CO) and H_2S were not detected. The level of CO_2 must reach above 5-10,000 ppm to impact poultry health and induce stress (Reece and Lott, 1980; Fernandes et al., 2014; Smith et al., 2016; NFACC, 2017). Such a high level of CO_2 is rarely achieved inside poultry houses. As poultry are monogastric animals, production of gases such as CH_4 in a poultry house is very minimal (Brouček and Čermak, 2015). All these facts suggest that NH_3 is a major pollutant among all the gases produced inside the poultry houses. Gases other than NH_3 are rather environmentally important as they contribute significantly to global warming and odor emissions (Brouček and Čermak, 2015).

2.4.1 Impact of NH_3 on the health of laying hens

Ammonia production is one of the major health concerns in the poultry house with poorly managed litter (Anderson et al., 1964; Hinkle, 2010). Ammonia at 25 ppm or above in poultry houses negatively impacts poultry by causing a reduction in feed intake and subsequently impeding growth rate of birds and decreasing production performance (Miles et al., 2004; David et al., 2015). Additionally, chronic exposure to 25 ppm or above can damage the mechanical defense system of the respiratory tract leading to increased susceptibility to secondary bacterial infections (especially *Escherichia coli* infection), Mycoplasmosis, Newcastle disease, and keratoconjunctivitis (Anderson et al., 1966; Miles et al., 2006). Ammonia, being a water-soluble gas, is absorbed in the litter as well as in mucous membrane of animals causing irritation to mucous membranes (Anderson et al., 1966; Nagaraja et al., 1984; Visek, 1984). Rarely, NH_3 inside the poultry house can reach as high as 50 to 100 ppm causing increased bird mortality (David et al., 2015).

Egg quality is also affected by elevated NH_3 concentration as indicated by reduced albumen height, increased albumen pH and liquefaction of albumen (Cotterill and Nordsog, 1954; Xin et al., 2011). NH_3 concentration at 50 ppm or above results in reduced feed efficiency and reduction in the number of egg-producing days (Charles and Payne, 1966; Deaton et al., 1984; David et al., 2015).

High levels of NH_3 in combination with high moisture can also cause a higher incidence of contact dermatitis, which includes mainly footpad dermatitis (FPD), and hock and breast burns (Berg, 2004). The lesions include hyperkeratosis and necrosis of the epidermis, with ulceration and inflammation of subcutis in severe cases (Ekstrand et al., 1997). These

lesions are sometimes referred to as “ammonia burns”. FPD is a major poultry welfare concern resulting from litter material with high moisture and NH_3 levels, which can lead to secondary bacterial infections and other complications (Mayne et al., 2007; Da Costa et al., 2014). Although considerable attention has been paid on FPD in broilers and turkeys (Shepherd and Fairchild, 2010), little attention so far has been given to FPD on laying hens (Niebuhr et al., 2009). Niebuhr et al (2009) reported FPD in 40% of laying hens per flock. The severity of FPD increases as litter moisture increases. FPD develops into hyperkeratosis, erosion, and discoloration of the skin ultimately resulting in ulceration of the area and possibly resulting in lameness and leg problems in severe cases. FPD, apart from negatively impacting chicken welfare, causes productivity loss by decreasing revenue generated from footpad export; however, this is more of a concern in broiler chickens (Shepherd and Fairchild, 2010).

2.4.2 Impact of NH_3 on poultry behaviour

It has been demonstrated that NH_3 influences the behaviour of laying hens (Kristensen et al., 2000; Drake et al., 2010; David et al., 2015). Ammonia is a chemical irritant, with the potential to stimulate the olfactory, gustatory, and chemosensory systems, particularly the trigeminal receptors of cranial mucous membranes, which triggers the activation of reflex and aversive responses (McKeegan et al., 2005). The aversive response elicited by NH_3 may include (but not limited to) fast blinking, gasping and head-shaking (Hughes, 1983; McKeegan, 2004; McKeegan et al., 2005).

Hens can notice NH_3 at or above 5 ppm and find it aversive at or above 20 ppm (NFACC, 2017). Therefore, the Canadian Code of Practice for Pullets and Laying hens has suggested

that corrective action must be taken to control NH_3 inside barn when it reached above 20 ppm (NFACC, 2017). When provided with a free choice in a chamber containing 0, 25 or 45 ppm of NH3, hens showed a relative preference towards the fresh air (0 ppm of NH_3) (Kristensen et al., 2000). Wathes et al. (2002) found that hens foraged, preened and rested significantly more in the fresh air than in 25 and 45 ppm of NH_3. Apart from laying hens, studies conducted in broiler chickens have also demonstrated the influence of NH_3 on their behaviour. Jones et al. (2003) observed that broilers were highly motivated to seek fresh air after exposure to 40 ppm of NH_3. Similarly, Jones et al. (2005) reported broilers avoiding NH_3 at concentrations of 20 and 37 ppm and suggested that NH_3 can be aversive at concentrations above 10 ppm. Another study conducted by Wathes et al. (2002) also showed that broiler chickens' occupancy and duration of visits were significantly lower in 20 and 40 ppm compared to 0 and 10 ppm of NH_3.

2.4.3 Impact of gases other than NH_3 to poultry

Limited studies have been conducted regarding the impact on behavioural response of hens in gases other than NH_3. Most of those studies are focused on the use of different gaseous levels for the stunning of poultry, especially CO_2 (Raj and Gregory, 1993; Webster and Fletcher, 2004). McKeegan et al. (2005) and McKeegan et al. (2006) reported display of behaviours such as headshaking, respiratory disruption, withdrawal, and ataxia[4] when birds were exposed to an increasing level of CO_2. Similarly, in a study conducted by Webster and Fletcher (2001), behaviours such as deep breathing and head shaking were observed in

[4] Ataxia refers to impaired movement and coordination

hens exposed to CO_2. When exposed to H_2S in a study by McKeegan et al. (2005), laying hens showed behaviours such as mandibulation, interruption of ongoing behaviours, eye shutting, and avoidance of H_2S.

2.4.4 Ammonia control strategies/Litter excreta management

Control strategies of NH_3, generally, can be divided into broad categories of ventilation practices, litter, and manure/excreta practices (selection and cleaning of bedding material, litter amendments, and managing build-up litter), and other practices such as oil and water spraying, and dietary manipulation (Wood and Van Heyst, 2016). This review is particularly focused on litter and excreta practices, litter amendments mainly Poultry Litter Treatment® and dietary manipulation among all other available strategies.

2.4.5 Litter and excreta practices

Practices of manipulating litter and excreta to reduce NH_3 in laying hen houses involves the use of appropriate litter material, management of used litter and use of litter amendments (Wood and Van Heyst, 2016).

Various studies have been conducted to study the impact of different litter materials on NH_3 emissions (Lien et al., 1998; Atapattu et al., 2008). Lien et al. (1998) reported lower NH_3 emissions from wood shaving compared to peanut hull litter. A study conducted in broilers by van Harn et al. (2012) found lower NH_3 emissions from maize silage compared to wood-shavings, wheat straw, and rapeseed straw. Similarly, Atapattu et al. (2008) observed significantly lower NH_3 emissions from refused tea as a litter material compared to sawdust and paddy husk. Differences in NH_3 emissions in different litter substrates is attributed to

their water activity (A_w), which is closely associated to the microbiological, physical and chemical dynamics of the substrates (van der Hoeven-Hangoor et al., 2014). Dunlop et al. (2016) found A_w of pine shavings to be similar to rice hulls and higher than peanut shells. The rapid fluctuations in A_w as a function of moisture may lead to different microbiological activity in various litter substrates (Dunlop et al., 2016), ultimately causing the difference in NH_3 emitted from those excreta-soiled litter substrates.

In addition to the use of appropriate litter material, management of used litter is critical in controlling NH_3 emissions. This includes frequent excreta removal/cleanout of soiled litter and management of litter moisture. In caged housing systems, the manure belt is located beneath the cage, which serves for collecting excreta from laying hens. Studies have indicated that frequent manure belt operation results in lower NH_3 emissions (Groot Koerkamp et al., 1995; Nicholson et al., 2004; Liang et al., 2005).

Another key factor in managing NH_3 emission is through the proper management of litter moisture. Keeping litter dry is a critical part of the litter management system to reduce NH_3 in laying hen facility. This can be achieved by drinker management, for example, using nipple drinker instead of bell drinker to lower litter moisture content by reducing water spillage (Elwinger and Svenson, 1996; Patterson, 2005). Also, litter moisture can be managed with proper ventilation in poultry houses (Patterson and Adrizal, 2005; Hinkle, 2010; Dunlop, 2017).

Litter amendment is another efficient method to inhibit NH_3 production in poultry facilities (Choi and Moore, 2008a). Several studies have reported the effectiveness of various litter

amendments, such as Poultry Litter Treatment (PLT®), ferric sulfate, Poultry Guard™, and alum (aluminum sulfate) (Pope and Cherry, 2000; Vicente et al., 2007; Choi and Moore, 2008b; Wood, 2015). These litter amendments work by inhibiting microbial growth and urease production and acidifying NH_3 to NH_4^+ (Choi and Moore, 2008a). Poultry Litter Treatment® has already been shown to be very effective in controlling NH_3 emissions (Terzich et al., 1998; Choi and Moore, 2008a; Wood, 2015). Poultry Litter Treatment® (sodium bisulfate, $NAHSO_4$) is a litter acidifier, which when applied to litter dissociates to produce hydrogen ion (H^+). Production of H^+ ion reduces the pH of litter. Reduced pH accelerates the reaction between NH_3 and H^+ to form NH_4^+. Once NH_4^+ is produced, it cannot volatilize to air thereby reducing NH_3 emission; however, NH_3 concentrations tend to increase again when pH increases (Li et al., 2006). Application of PLT® has been found to significantly improve litter quality, thereby improving the health and welfare of poultry (Terzich et al., 1998).

2.4.6 Dietary practices to reduce NH_3 emission

Dietary manipulation is one of the promising methods to reduce NH_3 in poultry houses (Li et al., 2012). This includes feeding low protein diet, optimum amino acid diets, fermentable fibrous diets (such as dried distiller grain soluble), and diets with acidifiers (Sutton et al., 2001; Roberts et al., 2007a; Li et al., 2012). Among these dietary strategies, the impact of reducing dietary protein on NH_3 emission has been extensively studied (Sutton et al., 2001; Bregendahl et al., 2002; Liang et al., 2005). Feeding amino acids to closely match bird's requirement is key in reducing nitrogenous waste from birds (Meluzzi et al., 2001). Reducing dietary protein and adding supplemental amino acids in the diet to closely match bird's

requirement has the potential to reduce NH_3 emission by 10 to 35% without impacting the performance of birds if amino acid requirements are met (Van der Peer-Schwering et al., 1997; Blair et al., 1999; Ferguson et al., 1998).

2.5 Key highlights of the literature review

- In laying hen houses, birds are exposed to the unnaturally high amount of excreta and litter/scratch pads soiled with excreta. Increased NH_3 production due to a large amount of excreta further exacerbates the situation. Exposure to excreta and NH_3 negatively impact the health and behaviour of laying hens.
- There are several strategies to manage excreta and reduce NH_3 emissions in laying hen houses. Such strategies include using unsoiled litter substrates such as wood-shavings, frequent removal of soiled litter, use of litter amendments such as PLT®, and dietary practices such as reducing protein.

2.6 Gaps in the literature

There were four major gaps identified while reviewing the literature. First, the majority of behavioural studies related to NH_3 in laying hens involve preference studies that only investigated hens' preference to different NH_3 concentrations, ranging from 0-45 ppm (Kristensen et al., 2000; Wathes et al., 2002). Additionally, the technique used to create NH_3 in a testing area involved use of an artificial source (Table 2.1), i.e., gas from an anhydrous NH_3 cylinder (Kristensen et al., 2000; Wathes et al., 2002; Jones et al., 2003; McKeegan et al., 2002). Often, the source of NH_3 is not mentioned (Charles and Payne, 1966; Jones et al., 2003). Pure NH_3 gas may not represent the actual aerial environment of laying hen

houses, which is the combination of various gaseous stimulants (e.g., CO_2, H_2S, N_2O, and CH_4 together with NH_3) with different sensory properties, along with dust particles and particulate matters continuously emitted from the environment in the hen house. Chapter 3 will aim to provide insights on how different concentrations of NH_3 obtained from different sources (both natural[5] and artificial) will affect behavioural responses in laying hens.

Table 2.1. Results from a selection of studies showing the behavioural response of NH_3 in poultry

Study	**Type of birds**	**Concentration of NH_3 (ppm)**	**Source**
Kristensen et al., 2000	Laying hens	0, 25 and 45	Artificial cylinder
McKeegan et al., 2002	Laying hens	2.5, 5, 10, 20, 40, 60, and 100	Cylinder bank (artificial)
McKeegan et al., 2005	Laying hens	5, 10, 20, 40 and 100	Artificial cylinder
Wathes et al., 2002	Broilers	0, 10, 20 and 40	Artificial cylinder
Jones et al., 2003	Broilers	0 and 40	Not reported
Jones et al., 2005	Broilers	4, 11, 20 and 37	Artificial cylinder

Second, studies to investigate whether hens prefer to avoid foraging on excreta are scarce. von Waldburg-Zeil et al. (2018) in their study reported hens consuming diets mixed with

[5] Natural source of NH_3 in this thesis is referred as NH_3 created from conspecific laying hen excreta

excreta. However, in the same study, the authors concluded that hens prefer feeding and foraging on the substrate without excreta suggesting that hens forage away from excreta when there is an opportunity. In laying hen houses, especially in enriched cages supplied with scratch pads and litter-based housing systems, hens are chronically exposed to excreta. Previous studies regarding scratch pad availability in the enriched cages have largely focused on the impact of the scratch pad on the expression of natural behaviours such as dust bathing and foraging, and health and performance of hens. However, no emphasis was placed on whether hens prefer or avoid the excreta-soiled scratch pads. Chapter 4 will aim to identify how exposure to excreta affects the behaviour of laying hens.

Third, preference studies conducted in the past have been focused on identifying hens' preference to different types of litter such as wood shavings, peat moss, sand, and straws. For example, Campbell et al. (2016) compared hens' preference for foraging or dustbathing on straws or shavings or no litter substrates. Further, Scholz et al. (2011) compared hens' preference for dustbathing in soft wooden pellets and wood shavings. The majority of these studies have emphasized the particle size of those litter substrates and their types (e.g. straw, wood shavings). Suitability of litter substrates has been associated with the friability of the substrate to ease dust bathing and other behaviours (Moesta et al., 2008). Whether or not the cleanliness of litter substrates or excreta-soiled litter affects hens' preference to these substrates has not been studied. To the knowledge of the author, there is no published information on whether laying hens prefer or avoid soiled litter substrates if provided with an opportunity to access unsoiled litter substrates or litter substrates that are treated with litter amendments such as PLT®. Chapter 5 will aim to address this gap.

Fourth, although reducing dietary protein is effective in limiting NH_3 emissions in laying hen houses, there is limited information available on the impact of such diets on hens' physiology and behaviour. Studies have indicated that low-protein diets, in conjunction with high energy, have negative impacts on the liver health of hens as such diets can contribute to the pathogenesis of fatty liver hemorrhagic syndrome (FLHS) in hens (Leeson, 2007; Jiang et al., 2013; Rozenboim et al., 2016; Robinson and Kiarie, 2019). Impaired liver function, due to fatty liver disorders, compromises the ability of liver to detoxify NH_3 (Dimski, 1994). This can lead to the accumulation of NH_3 in blood. Additionally, the level of plasma hepatic markers indicating liver damage [e.g. aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)] also gets elevated when liver health is compromised (Diaz et al., 1999; Yousefi et al., 2005; Zhang et al., 2008; Shini, 2014; Rozenboim et al., 2016). The resulting high level of NH_3 in blood can have toxic effect on central nervous system (CNS). Although the mechanism by which NH_3 impact CNS is controversial, studies have indicated that this involves structural changes in blood-brain barrier (BBB), effect on electrophysiological properties of CNS, interference with neurotransmitters and CNS biochemical pathways (Meijer et al. 1990; Dimski, 1994; Skowronska and Albrecht, 2012). Studies in rats and humans have indicated that the toxic effect of NH_3 can lead to altered brain function and subsequent changes in behaviour (Aguilar et al., 2000; Norenberg et al., 2005; Braissant et al., 2013). Studies (Apelqvist et al., 1999; Aguilar et al., 2000) have found that hyperammonaemia leads to the impairment of behavioural tasks, learning, spatial memory,

and reversal learning in rats. Chapter 6 will aim to identify the impact of reduced dietary protein on liver health, plasma hepatic markers, and learning of laying hens.

2.7 Objectives

The chapters of this dissertation are intended to contribute to the research in laying hens' behaviour and physiology. The general objectives are to understand the behaviour and health of laying hens in excreta polluted/ammoniated environments. The specific objectives of each chapter are:

- Chapter 3 – To investigate whether laying hens show different behavioural responses in NH_3 environments created from excreta (naturally-sourced) and gas cylinders (artificially-sourced). These findings will provide new approaches for the behavioural testing of laying hens in ammoniated environments and provide a basis to identify the concentrations of NH_3 that are aversive to laying hens.
- Chapter 4 – To investigate how laying hens respond to the clean compared to excreta-soiled scratch pads in enriched cages and how hens allocate their time towards performing different behaviours on clean and excreta-soiled scratch pads. These findings will provide information about the response of hens exposed to excreta on scratch pads.
- Chapter 5 – To investigate laying hens' relative preference for soiled litter (wood-shavings), unsoiled wood-shavings, PLT® treated soiled litter, and no litter substrates.
- Chapter 6 – To understand the impact of low-protein energy-rich diets on body weight, plasma hepatic markers and learning abilities in laying hens.

Chapter 3

3 Laying hens behave differently in artificially and naturally sourced ammoniated environments[6]

[6] A version of this chapter was published in Poultry Science with the following authors: Pokharel, B. B., V. M. dos Santos, D. Wood, B. Van Heyst, A. Harlander-Matauschek. https://doi.org/10.3382/ps/pex273.

3.1 Abstract

Laying hens are chronically exposed to high levels of ammonia (NH_3), one of the most abundant aerial pollutants in poultry houses. Tests for aversion to NH_3 in laying hens have used artificially sourced air/NH_3 gas mixtures (i.e. from a gas cylinder) showing that birds prefer fresh air to NH_3. However, artificially sourced air/NH_3 gas mixtures may not accurately reflect barn air conditions, where excreta emits a variety of gases. This study investigated whether laying hens differentiate between artificially and naturally sourced air/NH_3 mixture and how exposure to NH_3 affects foraging and aversive behaviours in laying hens. A total of 20 laying hens were exposed to artificially sourced [A] (from an anhydrous NH_3 cylinder) and naturally sourced [N] (from conspecific laying hen excreta) gas mixtures. Hens were exposed to A and N mixtures with NH_3 concentrations of 25 and 45 ppm, as well as fresh air [FA]. During the experiment, all birds were exposed to each treatment three times using a custom-built Polycarbonate chamber, containing a foraging area (containing raisins, mealworms, and feed mix) and a gas delivery system. All testing sessions were video recorded, analyzed with INTERACT® software and subjected to a GLIMMIX procedure in SAS. Our results showed that the laying hens spent less time foraging overall ($P < 0.001$) and were slower to commence foraging ($P = 0.004$) in ammoniated environments compared to treatment FA. Laying hens were more likely to forage for a longer time (with fewer interruptions) in N than in A treatments ($P < 0.001$). Laying hens also reacted with a greater aversion towards treatment A compared to treatment N ($P < 0.001$). These findings suggest that laying hens in our study behaved differently in artificially and naturally sourced air/NH_3

mixtures. This may be due to the presence of other excreta gases (e.g. H_2S, CO_2, and volatile odorous compounds), which may be a more familiar stimulus. These findings have implications for recommendations regarding NH_3 levels inside the poultry barn.

Keywords: laying hen, ammonia, excreta, foraging behaviour, aversive behaviour.

3.2 Introduction

Laying hens generally digest feed rapidly and subsequently eliminate waste quite frequently due to their high metabolic rate, among other physiological factors. It has been reported that a single laying hen can produce an average of 73 kg/1000 kg live weight of fresh excreta per day (Overcash et al., 1983). A number of different gases, as well as particulate matter, are produced by laying hens along with the excreta, some of which contribute to the characteristic odor of excreta. The most abundant aerial pollutant in poultry barns is ammonia (NH_3), which is produced by the bacterial decomposition of uric acid and undigested proteins from laying hen excreta (Kristensen et al., 2000; Liang et al., 2005; Fabbri et al., 2007; Kilic and Yaslioglu, 2014).

Chemical irritants, such as NH_3, in addition to the other gas components of excreta, have the potential to stimulate the olfactory, gustatory and chemesthetic systems, particularly the trigeminal receptors of the cranial mucous membranes, which trigger the activation of reflex and aversive responses (McKeegan et al., 2005). Contrary to previous misconceptions that birds have relatively poor chemical senses, various studies have established that poultry species are, in fact, capable of chemoreception and odor detection, naturally using chemical communication to regulate behaviour with regards to social interaction and predator avoidance, among other things (McKeegan et al., 2005; Widowski, 2010; Zidar and Løvlie, 2012). McKeegan (2004) reported irritation response in trigeminal receptors as a result of exposure to NH_3. The irritation caused to trigeminal nerves may mediate an aversive response to the elevated NH_3 concentrations emitted by their excreta (McKeegan, 2004).

Apart from laying hens, the response of trigeminal nerves to NH_3 has been reported in other animals such as guinea pigs (Sekizawa and Tsubone, 1994).

In an industry setting, the ability of a domestic laying hen to avoid its excreta is greatly limited. The resulting chronic exposure of laying hens to high levels of NH_3 and other gases, which would never be encountered in outdoor habitats, compromises their welfare in different ways (Wathes et al., 2002; Dawkins et al., 2004; Bessei, 2006). Elevated concentrations of NH_3 in poultry houses can reduce feed intake, impede growth, decrease egg production, damage mucous membranes of the eye and respiratory system, and increase susceptibility to *E. coli* infection, Newcastle disease, and Mycoplasmosis (Yahav, 2004; David et al., 2015). Changes in olfactory sensitivity and negative health outcomes of laying hens followed by exposure to NH_3 influence their behaviours (Schiffman and Nagle, 1992; Jones et al., 2000). The resulting response may involve increased vigilance, startling, head-flicking and freezing similar to the responses shown by birds against stimuli that are aversive to them (Hughes, 1983; Webster and Fletcher, 2004; Zidar and Løvlie, 2012).

The maximum concentration of NH_3 in poultry houses that is commonly considered to be acceptable is 25 ppm (Widowski, 2010), but this is a greater reflection of worker health and safety guidelines as opposed to animal welfare. This is despite the wide body of evidence that laying hens find exposure to NH_3 aversive at concentrations experienced regularly in a commercial barn scenario. Kristensen et al. (2000) and Wathes et al. (2002) reported that laying hens when given a free choice between fresh air, 25 ppm NH_3 and 45 ppm NH_3, preferred to stay in areas of fresh air compared to areas with 25 and 45 ppm of NH3. Behavioural changes in laying hens suggested a threshold for aversion to NH_3 at or below

25 ppm (Kristensen et al., 2000). In a study to measure behavioural motivation by Jones et al. (2003), the experimental birds demonstrated a consistent demand for fresh air after their exposure to 40 ppm of NH_3. Specifically, chickens exited the elevated NH_3 environment, suggesting that they were motivated to seek fresh air, evident by the fact that the door which they had to push through was heavily weighted.

The majority of behavioural studies done on the impact of gaseous emissions on laying hens have focused on how different concentrations of NH_3 affects the preference of birds (Kristensen et al., 2000; Wathes et al., 2002). Also, in most behavioural studies, the methods used to create a gaseous elevated NH_3 environment in an experimental setup involve the use of an artificial source of gas, i.e. gas from an anhydrous NH_3 cylinder (Kristensen et al., 2000; Wathes et al., 2002; Jones et al., 2003). However, the use of pure NH_3 gas from an anhydrous cylinder may not be an accurate predictor/representation of the gaseous environment inside laying hen barns, which involves the combination of various gaseous stimulants (e.g. CO_2, H_2S, N_2O, and CH_4) with different sensory properties and dust particles continuously emitted from excreta materials.

This study investigated whether laying hens demonstrate NH_3 avoidance and differentiate between NH_3 from excreta and from gas cylinders at specific concentrations [either 25 ppm or 45 ppm - these concentrations are based on the previous studies and on the history of common occurrence in poultry farms (Kristensen et al., 2000; Wathes et al., 2002; Jones et al., 2003; David et al., 2015)], or fresh air from an outdoor environment, when foraging in an experimental apparatus. It was hypothesized that higher NH_3 concentrations (25 or 45 ppm) from either type of source will result in delayed foraging (searching for food), less time spent

foraging, reduction in foraging bout lengths and increased time spent avoiding those concentrations. It was also hypothesized that laying hens will respond differently to elevated NH_3 environments created artificially or naturally, from an anhydrous NH_3 cylinder or from laying hen excreta, respectively.

3.3 Materials and methods

3.3.1 Ethical statement

The research protocol was approved by the University of Guelph Animal Care Committee (AUP#3169) prior to the start of data collection.

3.3.2 Animals and Husbandry

A total of 20 adult laying hens (*Gallus gallus domesticus*) of breed Dekalb White at the age of 57 weeks were used for this study. The birds were housed in windowless, well-ventilated home pens (1.8 x 2.5 x 2.9 m^3) consisting of four elevated platforms and perches during the entire laying period. Housing conditions were unchanged during the experimental period. A round feeder and nipple drinkers were provided. The floors of the pens were covered in wood-shavings. Lighting and temperature schedules were provided as per industry management guidelines. The average daily concentration of NH_3 in home pens was 1.27 ± 0.89 ppm, which was measured twice a day over a period of 30 days during the experimental period, using hand-held data logging NH_3 meters (Model ZD-800, Environmental Sensors Co., FL, USA).

The laying hens were fitted with silicone “backpacks” (consisting of two silicone squares (14.5 cm × 6 cm × 0.2 cm; 56 g)) for identification purposes on the back of the birds, and

two soft flexible, plastic clothes-line wires that wrapped around the wings and attached to the silicon squares by eyelets (for more details, please see Kozak, 2016). Each backback was randomly assigned to the numbers from 1 to 20.

3.3.3 Experimental Apparatus

A custom-built polycarbonate chamber (1 × 0.4 × 0.76 m^3) was used in the experiment (Figure 3.1). The chamber consisted of two atmospherically sealed compartments: a holding compartment (0.4 × 0.4 × 0.76 m^3) and a testing compartment (0.6 × 0.4 × 0.76 m^3). The size of the testing compartment was approximately double the housing requirements for non-cage housing system as recommended by NFACC (2017). The holding compartment led to the testing compartment through a guillotine door, operated by a rope. The testing compartment was provided with the feeder box, which contained an equal amount of the mixture of feed, raisins, and mealworms during each test. Below the testing compartment was the manure and gas compartment (0.6 × 0.4 × 0.15 m^3). The manure and gas compartment and the testing compartment were connected through small holes present throughout the floor of the testing compartment. A video camera (JVC GC-PX100), fitted to the side of the testing compartment, was connected to an iPad, which was used to operate the camera without disturbing hens during experimental conditions.

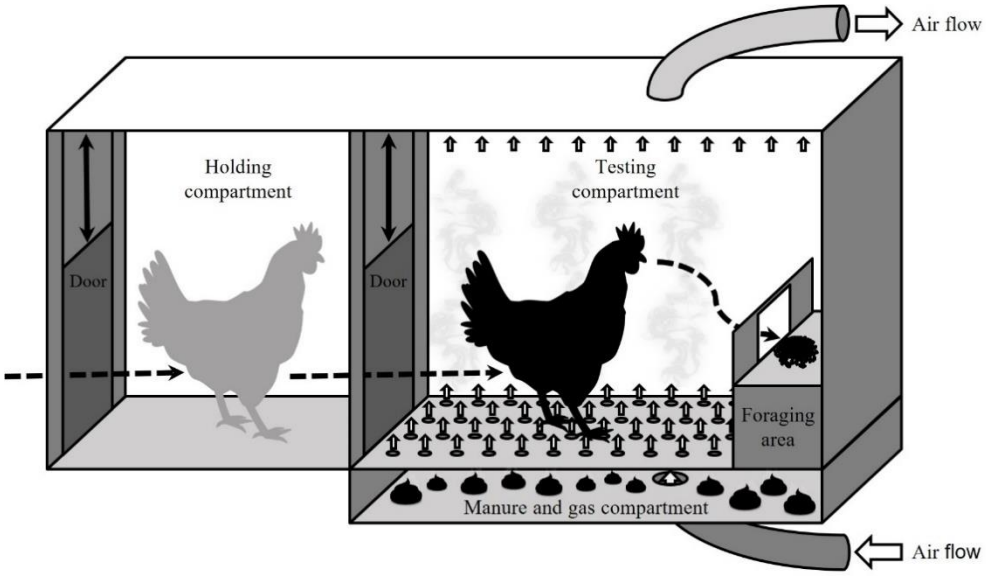

Figure 3.1. Two chamber test apparatus (side view, not to scale)

A climate-controlled mobile trailer, housed outside the layer barn, contained the NH_3 analyzer along with NH_3 gas cylinder containing 100 ppm of NH_3 balanced with air. A pressure regulator and a solenoid valve, controlled by a data logger, were used to supply NH_3 at 25 and 45 ppm of NH_3 to the test chamber. The data-logger code was designed to use the analog output from the NH_3 analyzer to determine when to open the solenoid valve, introduce gas into the testing compartment, and maintain the NH_3 concentration at an appropriate level. Ammonia concentration was continuously monitored using a chemiluminescent NH_3 analyzer (Model 17C, Thermo Electron Corporation, Franklin, MA, USA). For the gaseous stimuli produced from laying hen excreta, specific amounts of fresh excreta (details discussed in section 3.3.4) were placed in the manure compartment, located below the floor of the testing chamber. The excreta were then allowed to sit until the NH_3

concentration reached the desired level in the testing compartment. A pump was used to draw air from the manure compartment below the feeder box and introduce it into the top of the feeder box. This served to create a circulation cell and allowed adequate mixing of air. Between the gaseous simulations, an adjustable proportion of the clean airflow was allowed to flow through the system. The remainder of the clean air and the air/stimulus gas mix was removed by an extraction fan. The calibration of the NH_3 analyzer was done on a weekly basis with a 25 ppm NH_3 calibration gas balanced with air. Sample air was drawn from the facility to the trailer through a heated sample line at 121ºC (Model 0723-100, Clean Engineering Inc.) to prevent condensation within the air stream prior to entering the analyzer.

3.3.4 Ammonia Gas produced from Excreta

For the gaseous stimuli produced from laying hen excreta, preliminary tests were conducted with laying hen excreta to determine the amount of excreta required to produce the desired concentration of NH_3 in a testing chamber. To be consistent, excreta was always obtained from the same group of laying hens. The donor laying hens were provided with a corn and soybean-based diet with 18% protein. During preliminary observations, both fresh excreta samples (collected daily) and excreta samples that were frozen overnight were used. Fresh excreta samples were more consistent in producing desired concentrations of NH_3 in the testing chamber. Moisture content in thawed excreta samples (which were frozen overnight) might have caused inconsistencies in the NH_3 level produced inside the testing chamber. In this experimental setup, it was found that an average of 590 and 690 g of fresh excreta

sample was required to produce 25 and 45 ppm of NH_3, respectively, in the testing chamber with steady-state taking approximately 45 min for each concentration of NH_3.

3.3.5 Experimental Procedure

Before actual testing, individual birds were gradually habituated to handling, the testing apparatus and the food reward (the mixture of feed, raisins, and mealworms) for one week. During the habituation phase, each bird was placed in the experimental apparatus to imitate the experimental time (described below) without experimental gases. In the testing phase, the experimental birds were exposed to different concentrations of NH_3 gas. The test gases included fresh air (FA - 0 ppm of NH_3), naturally sourced air/NH_3 [N] mixture (supplied from laying hen excreta) and artificially sourced air/NH_3 [A] mixture (supplied from NH_3 cylinder) both consisting of 25 ppm and 45 ppm of NH_3. No auditory or visual cues were present to alert birds to the onset and end of the stimulus. The birds were unable to see the experimenter and gas delivery apparatus during the testing phase. On experimental days, metal crates with wheels were used to transport hens between their home pens and the testing room. Adequate feed and water along with wood-shavings on the floor of metal crates were provided while hens were waiting to get exposed to the treatment.

Testing began at 10 am each day by when the birds had finished laying eggs. Each of the 20 birds underwent three testing sessions for each treatment on separate days, producing a total of 60 responses to each type of gas at each concentration. The experiment lasted for a period of 30 days. A minimum of 30 min was allowed between each testing session to prepare for the next treatment. Treatments were presented systematically to the birds in a crossover design. To minimize the potential carryover effects, individual birds were never

tested on consecutive days and the intervals between each testing session for each treatment was at least one week.

Once the desired concentration of gas was achieved in the testing compartment, an individual bird was placed in the holding compartment, which provided them with a clear view of the testing compartment. After five seconds in the holding compartment, the guillotine door was opened, allowing the bird to enter to the testing compartment. Once a bird placed both of its feet in the testing compartment, the guillotine door was immediately closed. The bird had a maximum of one min to enter the testing compartment. Refusal to enter the testing compartment within one min was considered a failure. The procedure was repeated if the bird failed for the first time. After that, new testing began with another bird. The bird remained in the testing compartment for five min and was then removed. The whole experimental apparatus was cleaned and disinfected with 70% propyl alcohol wipes and air-dried (Walsh et al., 2013) and food reward was replenished to prepare for the next test batch.

3.3.6 Behavioural Observations

Video recordings of behaviours that occurred during each five min testing session were analyzed using INTERACT® software (Mangold International GmbH, Graf-von-Deym Str. 594424 Arnstorf, Germany). Preliminary observations helped to recognize and identify the kinds of behavioural responses likely to be seen during testing sessions. The video analysis was conducted by a person who was blinded with respect to the treatment status of each bird. The following behaviours were recorded: latency to enter the testing compartment, latency to commence foraging (i.e. feeding or pecking at food in the foraging area), the mean

length of foraging bouts and “aversive responses” including behaviours such as startling (sudden movement accompanied by frantic flapping of wings and jumping), vigilance (alerting movements with rapid jerky head movements), freezing (cessation of previous behaviours with no apparent movement of the body) and head-shaking (short and vigorous flicking of the head) (McKeegan et al., 2005; Zidar and Løvlie, 2012).

3.3.7 Statistical Analysis

Data were analyzed using the SAS (v9.4, SAS Institute Inc., Cary, NC, USA) statistical software package. Response variables used were (1) percentage of total time spent foraging, (2) mean length of foraging bout, (3) latency to enter testing compartment, (4) latency to start foraging and (5) aversive responses. The assumptions to the analysis of variance were confirmed using scatterplots of studentized residuals and a Shapiro-Wilk test of normality. The mean length of a foraging bout, latency to start foraging, and aversive response were fit with a log link to achieve normality and random distribution of residuals. Means and standard errors on the data scale were obtained using the Glimmix ilink option.

The analysis of behavioural data was conducted by a PROC GLIMMIX procedure. The model included behaviour performed as a dependent variable, treatment as a fixed effect and bird ID as a random effect. The Kenward-Roger approximation was used to calculate degrees of freedom. A set of preplanned orthogonal contrasts was applied to analyze the difference between A and N treatments, whereas least-square means were compared with a Tukey test. All results are presented as least square means ± standard error of the mean (SEM). Statistical significance was considered at $P < 0.05$ in all cases.»

3.4 Results

The source of NH_3 affected the success of achieving a steady state of NH_3 concentration in the testing chamber. For ammoniated environments created from excreta (N), steady-state of NH_3 concentration was achieved after approximately 45 minutes of placing excreta in the manure and gas compartment, whereas for ammoniated treatment created from artificial source (A), steady-state was achieved within 5-10 minutes.

All laying hens entered the testing compartment and latency to enter the testing compartment was not affected by treatment. Laying hens showed behavioural differences when exposed to environments with different NH_3 concentrations. Birds spent more time foraging ($P < 0.001$) in FA, compared to ammoniated treatments. No difference (Figure 3.2; $P = 0.46$) in foraging time was observed between treatments N and A. The mean length of foraging bouts was longer (Figure 3.2; $P < 0.001$) in treatment N compared to treatment A. The mean length of foraging bouts was not different (Figure 3.2; $P = 0.154$) between FA and 25 ppm air/NH_3 mixture from excreta, however, exposure to 25 ppm air/NH_3 mixture from an artificial source reduced (Figure 3.2; $P < 0.001$) the mean length of foraging bouts.

Although birds took longer (Figure 3.2; $P = 0.004$) to commence foraging in 25 ppm of NH_3 polluted environments compared to FA, no difference was observed with respect to latency to start foraging ($P = 0.946$) between N and A treatments.

Aversive responses displayed by birds was significantly affected by NH_3 concentration inside the testing area. The duration of time showing aversion-related behaviours was shorter (Figure 3.2; $P < 0.001$) in FA compared to NH_3 polluted environments. Birds showed

aversive responses for longer periods of time (Figure 3.2; $P < 0.001$) in treatment A compared to treatment N.

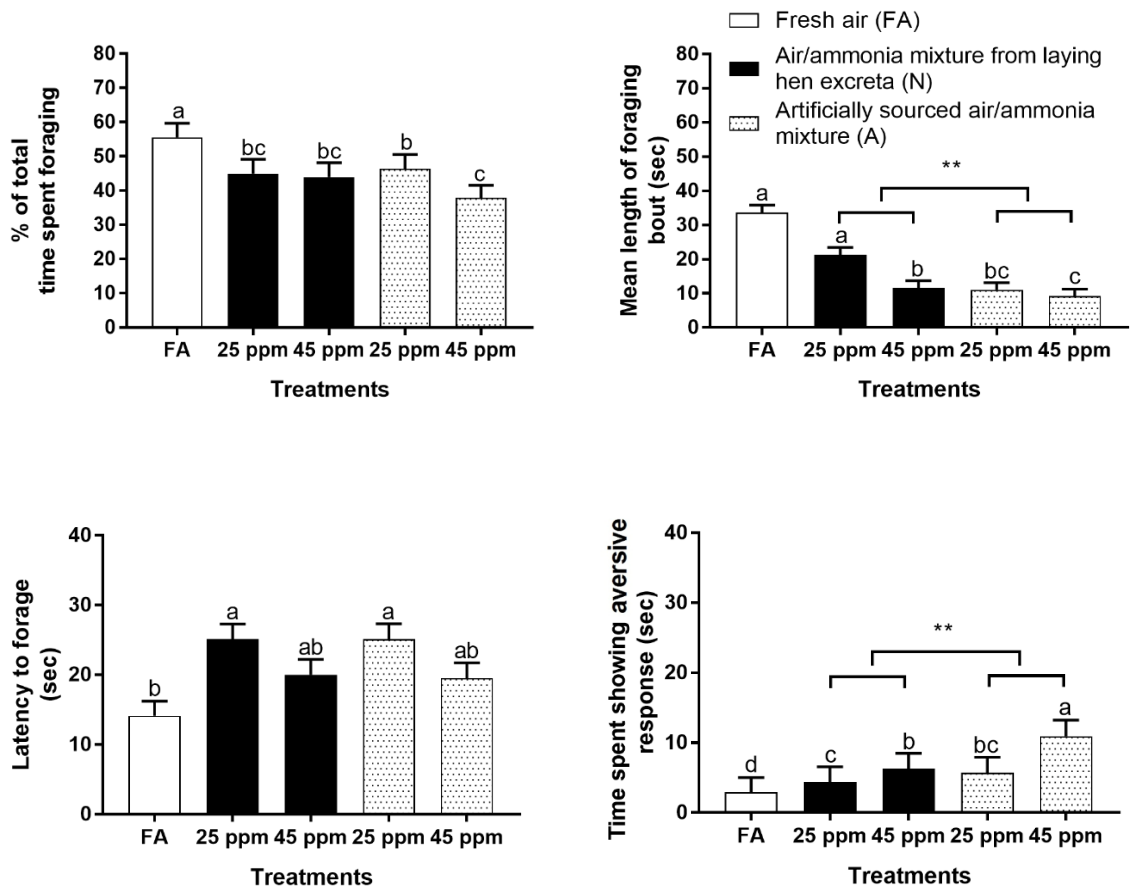

Figure 3.2. Behavioural response of laying hens to fresh air and different ammoniated environments (natural and artificial). Error bars represent standard error of the mean. Different letters (a to d) represent treatments that differed significantly ($P < 0.05$), and asterisks represent significant difference between naturally and artificially sourced air/NH_3 mixture ($P < 0.01$).

3.5 Discussion

From the present experiment, it was found that laying hens are able to detect ammoniated environments and are also able to differentiate between ammoniated environments produced from laying hen excreta and those from an anhydrous NH_3 cylinder. To the

knowledge of authors, no other studies compared behavioural responses of fowl to two different kinds of ammoniated environments, as the previous studies of this kind have been conducted using artificially sourced NH_3 (Jones et al., 1998; Kristensen et al., 2000; McKeegan et al., 2005). The results from this study are likely to be the most reliable to date in predicting responses of birds to naturally sourced ammoniated environments.

Birds spent more time foraging/searching for food in fresh air compared to ammoniated environments. This result is consistent with previous studies (Johnson et al., 1991; Smith et al., 1996; Kristensen et al., 2000). Under normal circumstances, birds spend large amounts of time foraging (Dawkins, 1989). Reduced foraging behaviour in gas-polluted environments, therefore, indicates that the motivation of birds to perform foraging behaviour is compromised. Although the proportion of time spent foraging was not different between artificially or naturally sourced NH_3, birds displayed a significant reduction in the mean length of foraging bouts in artificially sourced NH_3 compared to naturally sourced one. Decreased willingness to continue feeding in the artificial sourced NH_3 might be due to increased vigilance in environments that are more aversive to the birds (McKeegan et al., 2006). Repeated withdrawal from the feeding bowl was observed by McKeegan et al. (2006) when birds were exposed to high concentrations of noxious gases. Birds have a strong motivation to seek fresh air after they are exposed to high levels of NH_3 (Jones et al., 2003). Wathes et al. (2002) observed that domestic fowl spend less time in atmospheres contaminated with NH_3. The continuous seeking of fresh air while foraging in ammoniated atmospheres results in frequent interruptions of foraging behaviour.

The results confirm that NH_3 acts as an aversive stimulus to laying hens. Lower frequency of aversive responses was observed in the fresh air, which is consistent with results from previous studies (Smith et al., 1996; Kristensen et al., 2000; Wathes et al., 2002; McKeegan et al., 2005). Animals display a series of actions against stimuli that are aversive to them (Mills, 2010). Behaviours such as vigilance, startling, head-flicking and freezing were used as an indicator of aversive responses in previous studies (Hughes, 1983; Webster and Fletcher, 2004; Zidar and Løvlie, 2012). Birds become more alert and vigilant after detection of stimuli which may be aversive or potentially dangerous (Zidar and Løvlie, 2012). Head-shaking is a behaviour suggestive of an alerting response (Hughes, 1983) and is thought to represent a coping response to disturbance and environmental change (Dunnington et al., 1984; Dunnington and Siegel, 1986). Freezing behaviour in animals is an instinctive response to possible danger (Bolles and Tiley, 1973; Ferrari and Ferrari, 1990). Artificially sourced NH_3 gas elicited significantly more aversive responses in birds than naturally sourced NH_3 gas. The discrimination between artificially and naturally sourced NH_3 is likely due to the presence of familiar odors in the naturally sourced gas mixtures. However, measurement of other components in the laying hen excreta was outside the scope of this study. Future studies are needed to investigate cues other than NH_3 in laying hen excreta that can influence the behaviour of hens.

The experimental birds took longer to commence foraging in environments contaminated with NH_3 compared to fresh air. Similar behaviour was observed in birds when exposed to stimuli that were indicative of possible danger (fecal cues associated with predators) in a study conducted by Roth et al. (2008). Birds took longer to commence foraging in 25 ppm

ammoniated environments (irrespective of the source) than fresh air. However, no difference between the fresh air and 45 ppm treatments was observed although there was a tendency for birds to take longer to start foraging in 45 ppm environments. In this experimental setup, a mixture of laying hen feed, raisins and mealworms was used as a reward. Before entering the testing compartment, birds were kept in the holding compartment, which provided them a clear view of the food reward through the transparent door. The positive anticipation of the reward might have influenced the decisions of the birds before they were aware of the presence of NH_3 in the testing compartment which is also evident from the result that latency to enter was indifferent irrespective of the treatment. In addition, it was observed that the upward movement of the guillotine door caused some birds to turn back as soon as the door was open although they eventually entered the testing compartment. Also, some birds did not consume any of the food rewards during the entire experimental period, causing inflation in the latency to commence foraging in those birds. Latency data obtained from this study suggest a careful examination of how time affects the behavioural response of birds to aversive stimuli in future studies.

Elevated levels of NH_3 has been shown to be correlated to the behaviours indicative of stress as shown by the increase in the level of stress hormone (Dawkins et al., 2004; Drake et al., 2010). This might be the reason behind higher interruptions of foraging behaviour and increased incidence of aversive responses in the birds exposed to ammoniated environments in our experiment. Exposure to NH_3 also interferes with olfactory function and perception in different ways. Birds are able to distinguish between different concentrations of NH_3 through the trigeminal nerve (McKeegan et al., 2004). Ammonia stimulates the

trigeminal and olfactory nerves causing vasodilation and increased mucous secretion, leading to decreased respiration rate (Mills et al., 1969; Armstrong and Luck, 1974; Farbman, 1992; Sekizawa and Tsubone, 1994). In addition, NH_3, a highly water-soluble gas, is also readily absorbed by the mucous membranes of the eyes, and the nasal and oral cavities (Leduc et al., 1992). At high enough concentrations, the resulting reaction causes alkali burns in the mucous membranes (Anderson et al., 1964). These physiological effects and the resulting discomfort influence behavioural responses of birds, as demonstrated by alterations in feeding/foraging behaviour and aversive reactions (Jones et al., 2000). The negative health outcomes could also lead to a reduced or altered sensory input (nasal and/or visual) from the environment, which might affect behavioural patterns.

In the current experiment, it was ensured that a continuous flow of air/ NH_3 mixture was achieved to maintain the desired concentration of NH_3 at the bird's level. Although the motivation of laying hens to leave environments contaminated with NH_3 was not measured, their aversion towards ammoniated environments was demonstrated through the higher interruptions of foraging (shorter foraging bouts) and display of avoidance responses. The use of a small and properly sealed chamber was important to ensure the correct level of NH_3 throughout the experiment, as rapid diffusion of NH_3 may interfere with the accurate estimation of desired concentrations.

One of the major challenges in this study was to achieve the desired concentration of ammonia, especially in the ammoniated environment created from excreta. There were fluctuations of NH_3 concentrations, which could have been due to source (excreta) effect that was difficult to notice and also due to the opening of guillotine door in between the

holding compartment and the treatment compartment. To overcome this limitation, sensor was placed at birds' head height to make sure that birds were getting appropriate concentration of NH_3. Construction limitations of these kinds of behavioural studies where researchers aim to understand behavioural response of animals through gaseous challenge present unique opportunity for future researchers to investigate the possibility of best possible test set up and arrangement.

In summary, laying hens prefer to forage in the fresh air and behave differently in naturally and artificially sourced NH_3. The same concentration of NH_3 generated from an artificial source was more aversive than if it was from a natural source, as depicted by changes in foraging bout lengths and increased occurrences of aversive behaviour. In the future, to understand whether the behavioural responses of hens were due to level of ammonia or source, studies could be conducted using additional odorous treatment from different sources (e.g. predator excreta odour). The results obtained from this study may provide new approaches for the behavioural testing of birds in ammoniated environments, since, until this study, no previous investigations used poultry excreta as a source of NH_3. Also, these findings imply that the maximum recommended concentration of NH_3 should be reconsidered carefully after complete characterization of the gaseous environment in the poultry barn. Further studies will be needed to identify different components of poultry excreta that may affect behavioural responses of birds. The results from this study can also be instrumental in reviewing the concentrations of NH_3 that are aversive to birds.

3.6 Acknowledgements

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Chapter 4

4 How does the presence of excreta affect the behavior of laying hens on scratch pads?[7]

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4.1 Abstract

Enriched cages for laying hens provide scratch pads for foraging and dustbathing on the wire mesh floors. Apart from foraging on scratch pads, hens also defecate on these pads, causing them to become soiled with excreta. This study was conducted to determine the relative preference of laying hens for foraging on clean (C) scratch pads or scratch pads soiled with excreta (E), and to study the behaviours performed by hens on such pads. A total of 288 laying hens were housed in 16 enriched cages (18 hens/cage), each divided into 2 compartments. On a daily basis, half of the scratch pads (one in each compartment) were removed and cleaned, while the other half were cleaned and then covered with 550 g (0.35 g/cm^2) of conspecific excreta. The C and E scratch pads were then put back into the cages in a systematic order to avoid side bias. Feed was delivered automatically onto the scratch pads as a litter substrate. The frequency of visits and the total time spent performing different behaviours on C and E pads were video-recorded [the time of video recording was relative to litter (feed) delivery on the scratch pads] for a total of 10 min/d, 3 times/wk, over a period of 4 wk. Overall, the allocation of the time budget for different behaviours was found to be - in order of greatest to least amount of time - resting, locomotor behaviours (walking and running), foraging, and dust bathing. Laying hens showed a relative preference for E scratch pads by visiting them more frequently ($P = 0.001$), and spent more time ($P = 0.035$) foraging on them, whereas they rested for more time ($P < 0.001$) on C scratch pads. The relative preference for E scratch pads during foraging signifies the innate importance of foraging substrates in enriched cages for laying hens. Similarly, the longer use of C scratch pads for resting indicates the preference for a clean resting surface in enriched cages.

Keywords: laying hens, scratch pad, excreta, enriched cages, foraging.

4.2 Introduction

Enriched cages have been designed to overcome some limitations of conventional cages to support behaviours that laying hens are highly motivated to perform (Weeks and Nicol, 2006). These cages provide a varying amount of horizontal space for locomotion and other behaviours such as nesting, perching, foraging, and dustbathing (Appleby et al., 2002; Lay et al., 2011). In enriched cages, foraging and dustbathing are typically performed on scratch pads, which are sections of synthetic turf of various designs and other artificial materials. These cages are also equipped with a mechanism which delivers a small amount of litter material, such as feed, onto these scratch pads, upon which hens can scratch, forage and dustbathe (Scholz et al., 2011).

However, not all farmers provide feed as a foraging and dustbathing substrate regularly, or otherwise provide it in small amounts, as feed represents the major cost of production for farmers (Lee et al., 2016). The result is that no litter substrate is delivered onto the scratch pads, and so little motivation is offered for hens to scratch, forage, and dustbathe upon them. Furthermore, defecation onto, and accumulation of, excreta on the scratch pads causes them to become soiled and unhygienic.

Poor scratch pad hygiene can become an increasing health threat and is, therefore, a welfare concern. Excreta on the scratch pads may be a vector for fungi, viruses, bacteria, and toxins (Himathongkham and Riemann, 1999; Zarrin et al., 2010; Imran and Ali, 2014), in addition to contributing to poor air quality, with high levels of dust and NH_3 (Turnbull and Snoeyenbos, 1973; Weaver and Meijerhof, 1991). Collectively, these downstream consequences of poor hygiene can lead to decreased feather cleanliness and give rise to

health issues such as ulcerative footpad dermatitis (i.e. bumblefoot) (EFSA, 2005). Furthermore, poor hygiene in enriched cages can be a food safety concern, as unclean conditions promote egg dirtiness and bacterial contamination of eggshells, which translate to an economic loss for farmers (Guinebretière et al., 2012). For these reasons, in addition to expensive cleaning techniques, farmers often choose to remove scratch pads from their cages or are reluctant to use them at all, which could jeopardize the welfare of laying hens housed in enriched cages.

While there are considerable disadvantages for hens and farmers when it comes to soiled scratch pads, there may be some hidden benefits associated with excreta contact as well. Despite the evidence that dirty scratch pads in enriched cages are a hygienic challenge, some studies have reported that certain wild bird species benefit nutritionally from foraging on and consuming excreta. McGowan (1995) observed that parent birds of many passerine species consume the excreta of their neonatal offspring shortly after hatching, due to the excreta's high nutritional value. In addition, other bird species have also been found to ingest the excreta of other animals, mainly for its nutritional benefits. Ungulate excreta, for example, contain high levels of intact carotenoids, which are essential for vertebrates and have pigmentary, as well as antioxidant and immunostimulant, functions (Negro et al., 2002). As a result, the Egyptian vulture, Neophron percnopterus, is commonly seen foraging on and consuming cow, goat, and sheep excreta (Negro et al., 2002). Apart from micronutrients, food (i.e. prey) items likely to be found in excreta may be another reason that animals forage on animal dung: hooded crows (*Corvus corone*) preferentially forage on fresh horse excreta as it is a reliable source of dung beetles (Horgan and Berrow, 2004).

For laying hens, however, the nutritional benefits of foraging on or consuming excreta, if any, are unknown.

In nature, wild red junglefowl (Gallus gallus) spend more than 60% of their time foraging in the natural litter (Dawkins, 1989). In commercial housing systems, domestic hens continue to demonstrate this high motivation to forage, even when they are provided with *ad libitum* feed, which hens can access at no cost (i.e. hens do not have to perform energy or time demanding operant tasks to access feed) (Bubier, 1996). In litter-based housing systems, laying hens scratch, peck and dustbathe in the litter substrate (Merril and Nicol, 2005), which is often unchanged over the entire duration of a laying period, leading to the accumulation of a considerable amount of excreta. In the case of laying hens housed in enriched cages, excreta and litter material (if provided) on the scratch pads may be the only foraging substrates available in the midst of a wire grid floor.

It is clear that the excreta-soiled scratch pads in enriched cages are undesirable from a hygienic point of view, and solutions to improve the health and welfare of laying hens kept in these housing systems should be addressed. However, there is a knowledge gap regarding how laying hens respond to excreta in the short and long term. As a first step to bridging this gap, this study was conducted with the objectives of examining how feed (litter substrate) on clean (C) compared to excreta-soiled (E) pads could influence hens' choice regarding where to forage, and once on the scratch pads, how hens allocate their time towards performing different behaviours on each type of scratch pad. As hens instantly started to defecate on the C pads, their preferences for C or E pads was considered 1 hour after they were put into the cages, in order to keep the choices distinguishable.

4.3 Materials and methods

4.3.1 Ethical statement

The research protocol was approved by the University of Guelph Animal Care Committee (AUP#3169) prior to the start of data collection.

4.3.2 Animals and Husbandry

A total of 288 laying hens of four breeds (White Leghorn, Rhode Island Red, Columbian Rock, and Barred Rock) were obtained from the University of Guelph Arkell Research Station at 25 wk of age. Hens were housed in enriched cages manufactured by FDI Cage Systems of Mitchell, Ontario (369 cm × 65 cm; 18 hens/cage, 4 cages/breed, 16 cages total). Each enriched cage (Figure 4.1) was partitioned into two identical compartments for the purpose of the experiment, and each individual compartment was equipped with a curtained nest area (107 cm^2/hen; red curtains), perches running length-wise (13.7 cm/hen), a scratch pad (details described in section 4.3.3), automatic feeders on the outside of the cage (10 cm/hen), and nipple drinkers (1.5 hens/nipple). Feed and water were provided *ad libitum*. The light period was 14L: 10D.

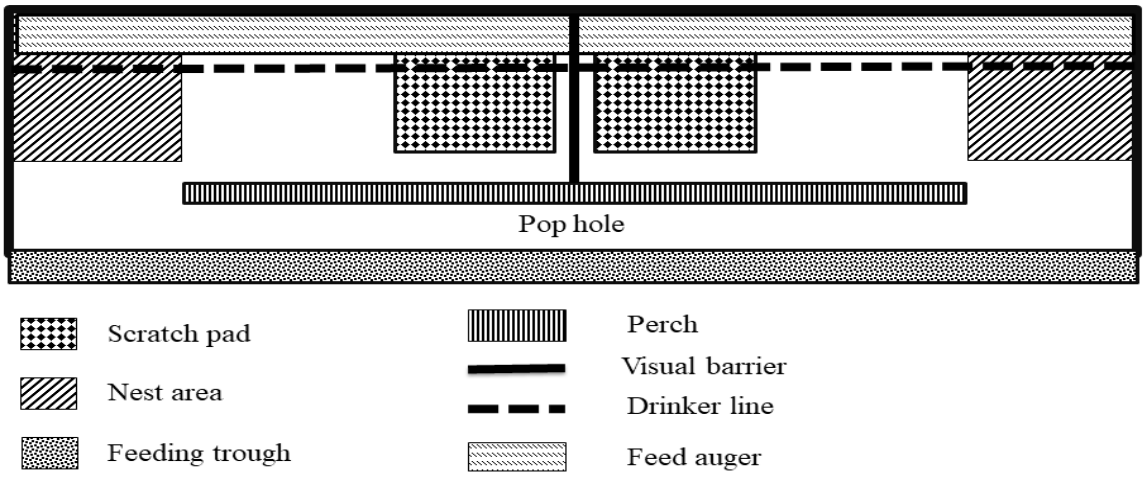

Figure 4.1. Schematic layout of an enriched cage for 18 laying hens featuring two identical compartments freely accessible through a pop hole (not to scale)

4.3.3 Experimental Design

Two identical compartments in each cage were joined by a pop hole to allow hens to move freely between compartments. A white plastic sheet (visual barrier) was added between the width and length of the two compartments, aside from the pop hole, to prevent the transfer of visual information between the cages and treatments. Each compartment contained one scratch pad (31 × 51 cm; 88 cm^2 /hen); together, the two scratch pads per cage occupied approximately 28% of the total surface area of the cage floor. The square scratch pads consisted of brown, synthetic turf (Astroturf®), which resembled natural grass in both appearance and texture. On a daily basis one hour (10:00 h) before starting video recording,

half of the scratch pads were removed and cleaned, while the other half were cleaned and then covered with 550 g (0.35 g/cm^2) of fresh conspecific excreta, which was determined to be the average amount of excreta produced in a day by 9 laying hens in the experimental conditions. Before actual testing, preliminary tests were carried out to determine the possibility of excreta falling through the holes of scratch pads; however, it was not the case due to the sticky nature of excreta. One C and one E pads were then put back into each cage in such a way that allocation of treatments across sides (right or left) was evenly spread. Feed (approximately six g/delivery) was delivered automatically for one minute onto both scratch pads by a spiral conveyor pipe (feed auger) every two hours. A pilot study identified that hens were most active on the scratch pads at 11:00 h, six hours after the lights were turned on. The video was recorded (including the time when feed was delivered) and analyzed for 10-min at 11:00 h, as soon as the feed was delivered from the auger onto the C and E pads, three times/wk, over a period of four wk (resulting in a total of 64 hrs of analyzed video recordings). As hens started to defecate on the clean scratch pads, all of the 10-min observation periods took place one hr after exposure of hens to the treatments. Also, due to the discrepancy of a few seconds created by feed delivery from one cage to the other, the time to start behavioural recording for each cage was carefully accounted for while analyzing the videos. Lastly, the laying hens were habituated to the experimental set-up (scratch pads and handling) over a period of 10 days before the video recordings began.

4.3.4 Behavioural measures

Continuous video recording of laying hen behaviour on the scratch pads was conducted at normal speed (25 frames/s) using video cameras (Samsung SNO-5084R, Samsung

Techwin Co., Gyeonggido, Korea) placed over each cage. The number of visits to C and E scratch pads (per min, 10-min total), the duration of the visits, and the duration of the behaviours (referred to in Table 4.1) for each group of hens were analyzed using INTERACT® software (Mangold International GmbH, Graf-von Deym Str. 594424 Arnstorf, Germany).

Table 4.1. List of mutually exclusive behaviours that occurred on scratch pads.

Behaviours	Description
Foraging	Scraping or pecking scratch pads with feet or beak, respectively
Dustbathing	Performing dustbathing elements such as bill-raking, wing-shaking, scratching, lying on the side and head-rubbing, as described by Kruijit
Resting	Sitting on the scratch pads, without any other activity
Locomotor	Walking or running on the scratch pads

4.3.5 Statistical analysis

The model was fitted using PROC GLIMMIX of the SAS System (v9.4, SAS Institute Inc., 165 Cary, NC, USA, 2016). The data analysis was based on a mixed modeling approach for randomized experiments with repeated measures (Piepho et al., 2004). To analyse the number of visits, foraging duration, dustbathing duration, resting duration and locomotor behaviour duration, a generalized linear mixed model was employed with the fixed effect of scratch pad treatment (C and E), breed (White Leghorn, Rhode Island Red, Columbian Rock, and Barred Rock) and treatment × breed interaction. The average bird weight per cage was used as a covariate and cage was used as a random effect. The number of visits

was fitted with a Poisson distribution, whereas all other variables were fit with a log link to achieve normality and random distribution of residuals. Means and standard errors on the data scale were obtained using the Glimmix ilink option. Due to the repeated measures taken on the same group of birds (cage) at different time points (days), an autoregressive covariance structure of order 1 was fitted to the cage-by-day effect. The degrees of freedom were adjusted using the Kenward-Roger method. All results are presented as least square means ± standard error (SE). Statistical significance was considered at $P < 0.05$ in all cases.

4.4 Results

The times allocated to various behaviours on both the C and E pads within the 12 x 10-min observation periods are presented as percentages in Table 4.2. In general, the most common behaviours laying hens performed on both the C and E scratch pads were resting, followed by locomotor behaviours, and foraging. Dustbathing occurred to a lesser degree. No eggs were laid on the scratch pads during the experimental period.

Table 4.2. Percentage of time spent performing different behaviours by laying hens on clean (C) and excreta-soiled (E) scratch pads during 10-min recording sessions over a 4 wk period.

Behaviours, %	Treatments	
	C	E
Foraging	21.5	25.0
Resting	46.7	43.0
Dustbathing	4.4	3.5
Locomotor behaviours	28.3	27.6

Hens visited the E pads more ($P = 0.001$; $F_{1, 376} = 18.03$) frequently than the C pads (3.6 ± 0.13 vs. 3.2 ± 0.13 visits/min). Hens spent a longer time ($P < 0.001$; $F_{1, 374} = 200.82$) resting on C pads compared to the E pads (Figure 4.2). No significant differences were observed with respect to dustbathing behaviour (Figure 4.2) and locomotor behaviours (Figure 4.2) between the C and E scratch pads. However, hens were found to forage for a longer time ($P = 0.035$; $F_{1, 155.5} = 4.74$) on the E pads compared to the C pads (Figure 4.2). Interestingly, while foraging on the E scratch pads, some of the hens were observed consuming excreta. There was no significant effect of breed, breed by treatment interaction, or average body weight on any of the behavioural responses.

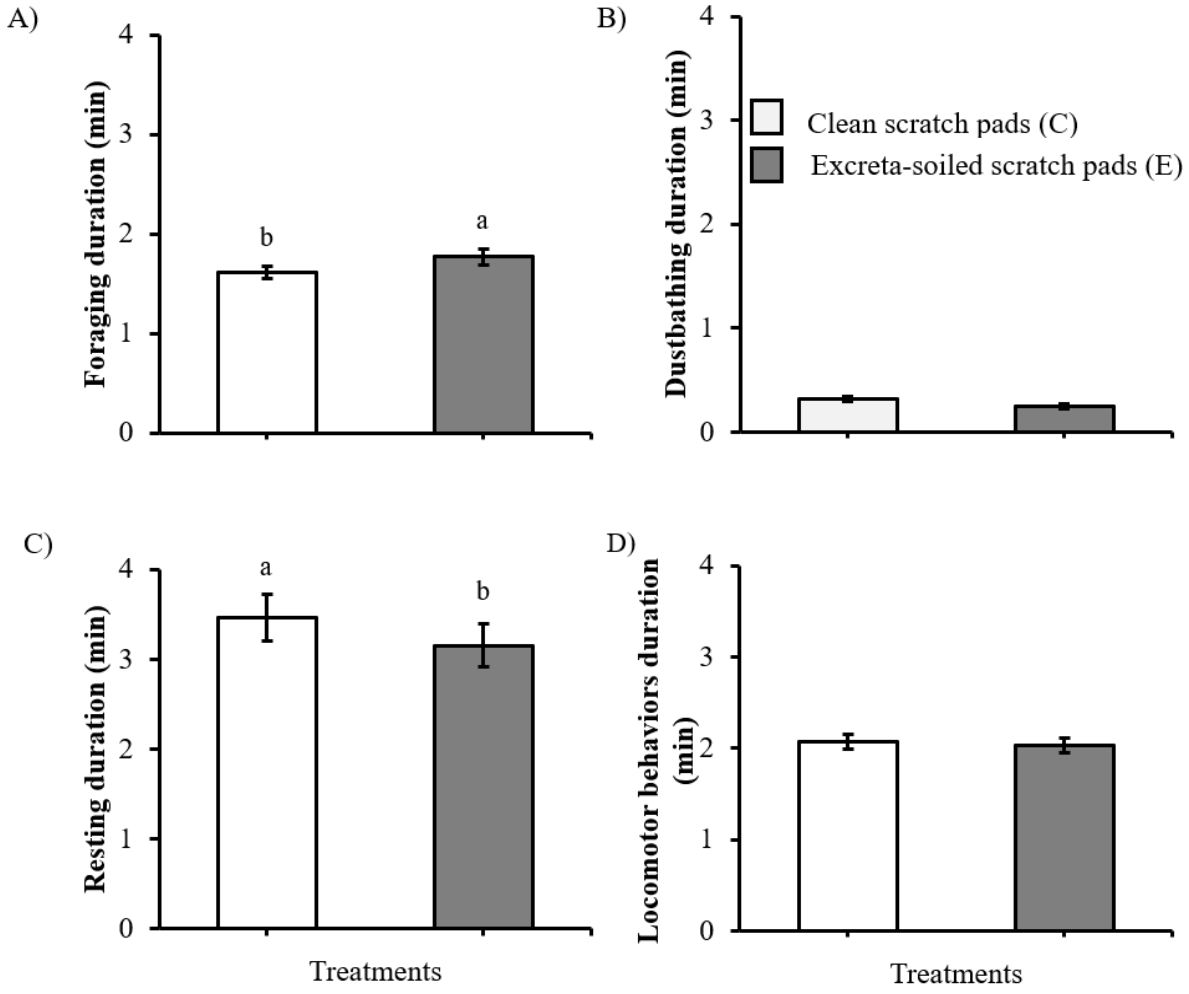

Figure 4.2. Mean Duration of foraging (A), dustbathing (B), resting (C) and locomotor (D) behaviours of laying hens on clean and excreta-soiled scratch pads out of 10-min observation period. Values represent least square means ± standard error. Different letters (a and b) represent treatments that differed significantly (unpaired t-test: $P < 0.05$).

4.5 Discussion

This study investigated whether laying hens behave differently on and if they have a relative preference for foraging on, scratch pads with or without excreta.

The results presented show that during the observation period (i.e. for 10-min commencing six hours after the lights were turned on), laying hens spent the greatest amount of time resting, followed by locomotor behaviours, foraging, and then at a much lower proportion, dust bathing. Moreover, some preferences existed with regards to whether a behaviour is performed on a scratch pad with or without excreta. It seems likely that these preferences are related to the physical and chemical aspects of the foraging material (feed or a mixture of feed and excreta) provided on the scratch pads and the behaviour that they promote. However, in this study, there was no provision to preclude olfactory and auditory modes of communication. Therefore, whether the hens were responding to visual cues of a feed-excreta mixture or whether olfactory (excreta odor) cues and/or acoustic cues (foraging sounds) may have played a role in the decisions of the hens was not distinguishable. In terms of the duration of foraging and the number of visits made to forage on the scratch pads, the laying hens preferred the scratch pads with the feed-excreta mixture, although the difference was small. The primary reason for this may be that the feed present on the clean scratch pads was rapidly removed by the eating and scratching activities of the birds. Thus, with feed no longer present, the hens' initial interest towards the scratch pads may have quickly waned. Fresh excreta allows feed to remain attached to and linger on the surface of the scratch pads, perhaps maintaining hens' interest for slightly longer periods of time or making it more difficult for feed to be extracted, or both. This greater interest and challenge posed by the scratch pads soiled with excreta may have resulted in the higher number of visits to and longer durations of time spent foraging on those pads, relative to those that were clean. However, it remains questionable whether the hens were responding to feed,

excreta or a combination of both. Additionally, the social environment (i.e. the presence of hens on scratch pads prior to the observational period) of the hens cannot be disregarded, which could have affected subsequent behavioural measurements.

In this study, hens showed a high degree of pecking and scratching behaviour on scratch pads with the feed-excreta mixture, despite the presence of *ad libitum* feed in the feeding trough. Several studies have indicated that animals expend additional energy to “work” for food, even if an identical quality and quantity is more easily available (Osborne, 1977; Inglis et al., 1997; Lindqvist et al., 2006; Harlander-Matauschek and Häusler, 2009). This may explain why hens preferred to forage more on the scratch pads on which excreta was mixed with feed particles, making it more difficult for the hens to obtain feed from the pads. This not only provides hens with an opportunity to perform additional work to obtain food but consequently, it may also increase physical and mental activity levels in hens, allowing them to actively interact with stimuli to gather information about the environment (Newberry, 1999). The higher level of foraging on the excreta-soiled scratch pads also demonstrates that hens are highly motivated to spend a portion of their time foraging.

Some of the hens were also observed consuming excreta from the scratch pads while pecking at them. Generally, there is a lack of information on the nutritional benefits of coprophagy in laying hens. However, it has been shown in wild birds that important micronutrients can be obtained by feeding on the excreta of other species, or that of their own (McGowan, 1995; Negro et al., 2002). The investigation of the nutritional components of laying hen excreta and their potential benefits to laying hens was not within the scope of this study, but the possibility that poultry excreta contain significant levels of relevant

minerals, such as potassium and phosphorous (Nicholson et al., 1996), could be considered in future studies.

Laying hens spent a significant amount of time (i.e. over one-third of the total observation period) resting on the scratch pads. The soft surface of the scratch pads is likely more comfortable to rest on than the bare wire floors of the cage (Reed et al., 1966), and their placements in the corners of the cage may have created a secluded area to which hens can retreat. In further consideration of the cage design, hens may have avoided resting on the perches, due to a high chance of being disturbed on them and chosen to rest on the scratch pads instead. Perches were located directly in front of the pop holes, which were used by the hens to travel between the cage compartments and to access the feed troughs (Figure 4.1). This may have resulted in high traffic levels around the perches, encouraging the hens to seek resting areas in a more secluded and distal location. Hens were specifically observed to rest for a longer duration on the clean pads, compared to the scratch pads with excreta, although the difference was small (3.5 vs 3.1 min). A possible explanation for this may be the higher frequency of foraging behaviours on the excreta-soiled scratch pads, which resulted in more social disturbances on those pads.

Hens were not found to have a relative preference for dustbathing on the clean scratch pads or the scratch pads soiled with excreta. Birds prefer to dustbathe in dry material (Scholz et al., 2011) and as such, hens were expected to prefer to dustbathe on the clean scratch pads with feed, as opposed to the pads with excreta, which were moist. However, as mentioned above, constant pecking and scratching may have resulted in the rapid removal of feed from the surface of the clean scratch pads. The resulting absence of feed as a dry, ideal

dustbathing substrate on the clean pads, in combination with the presence of excreta as a less attractive substrate on the excreta-soiled pads, may be a reason for the lack of a significant difference in dustbathing behaviour. Additionally, dustbathing was observed to a much lesser degree compared to other behaviours. Laying hens generally do not dustbathe on a daily basis, and the scratch pads may have been too small for this behaviour to be performed more often (Vestergaard, 1982; Lindberg and Nicol, 1997; Alvino et al., 2013; Louton et al., 2016). Furthermore, although dustbathing behaviour may occur at any time of day, hens tend to show peak dustbathing in the afternoon (Campbell et al., 2016; Vestergaard et al., 1990). This study was conducted in the late morning, which might also have influenced the dustbathing results.

Although this short-term study cannot predict relevant long-term effects on the behaviour of laying hens kept in enriched cages, it generates important findings. Firstly, while farmers add scratch pads to cages with the intention of providing hens with a foraging and dustbathing area, the scratch pads in this study fulfilled a multifunctional purpose, as shown by the percentages of time spent performing different behaviours on the pads. Secondly, laying hens were found to have a relative, albeit small, preference for foraging for feed on excreta-soiled scratch pads, compared to clean pads. While the intention of this study is not to recommend the addition of scratch pads soiled with excreta to enriched cages based on this relative preference, it does underline the strong motivation of hens to forage in response to the environment in which they live, even if that environment features a substrate (e.g. excreta) that humans find inappropriate, or that some herbivorous animals would avoid (Cooper et al., 2000). This raises another important and unanswered question: what are the

short- and long-term consequences of foraging upon or ingesting excreta on the behaviour of laying hens, especially in consideration of the shift from conventional cages to alternative housing systems? Although indications of discomfort due to contact with excreta in alternative housing systems may not be very severe or obvious, the duration of exposure to excreta and its consequences on avian behaviour and physiology is relevant to animal welfare and should be investigated in the future.

4.6 Acknowledgements

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Chapter 5

5 Do laying hens differentiate among excreta-soiled and unsoiled litter substrates?

5.1 Abstract

In commercial non-cage housing systems, laying hens may lack access to litter substrate covering the floor or may have access to litter substrate which has not been replaced since first access (soiled/build-up litter). To find out if litter substrate is important to the laying hens themselves, the present study investigated to what extent laying hens will work (by pushing a weighted door) to access unsoiled/fresh litter, soiled litter, soiled litter treated with Poultry Litter Treatment (PLT®) or no litter covering the floor, and compared it to the motivation to access feed. A total of 84 laying hens were housed in six mixed-strain pens with the same number of brown-and white-feathered hens in each group. Each of the pens was partitioned into two identical compartments: a home (H) and a treatment (T) compartment, connected by two one-way push doors. Treatments were 1) S: soiled wood-shavings, 2) PLT®: soiled wood-shavings treated with a litter acidifier (sodium bisulfate), 3) US: unsoiled clean, wood-shavings and 4) NS: no substrate. Their motivation towards litter substrate treatment was compared to the motivation to access fresh feed (F). The doors that led to T compartment weighed 0, 20, 40 or 60% of the hens' average weight. Treatment and door weight combinations switched between pens after each testing cycle (8 days). Testing took place over 40 days. Birds' presence in each compartment was determined via radio-frequency identification to automatically identify and track tags attached to the hens over 23 hours per day. The effect of treatment, push door weight and their interaction on latency to enter treatments, the number of visits and time spent in treatments was analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS V9.4). Hens worked at least as hard to access litter substrates as they did to access the feed. Hens showed a relative preference

for substrates over no substrates and did not show a strong general preference for one of the substrates (S, US or PLT®) provided. Hens' motivation for litter substrate on the floor is consistent with earlier results and shows that litter substrate is important to the laying hens.

Keywords: laying hens, excreta, PLT®, bedding, motivation, push door.

5.2 Introduction

In cage-free laying hen houses, litter substrates help to keep birds clean and dry (Guinebretière et al., 2012). Litter substrates/bedding binds moisture from excreta and reduces odor (Garcês et al., 2013). Additionally, bedding material serves as foraging, nesting and sleeping/resting material (Cooper and Albentosa, 2003; Campbell et al., 2017). When kept in commercial housing systems, laying hens may lack access to litter substrate (hens are kept on wire or slatted floors) or may have access to litter substrate which has not been replaced since first access (Van Staaveren et al., 2018). In this case, laying hen litter is a combination of excreta, wasted feed, feathers, dust and/or bedding material on the floors of bird housing systems (NFACC, 2017). In these systems, birds may be exposed to excreta and excreta gases that would not arise in their natural habitats (Whates et al., 2002), as litter generates NH_3 as a by-product of microbial excreta digestion (Groot Koerkamp et al., 1998). Excreta exposure routes include oral (Pokharel et al., 2018; von Waldburg-Zeil et al., 2018), dermal (Kaukonen et al., 2016), inhalation (Nunes et al., 2016) and combinations thereof. All can adversely affect laying hen behaviour (Pokharel et al., 2017) and health (Anderson et al., 1964; Nagaraj et al., 2007).

Excreta gas is not only a source of greenhouse gases and dust but also a source of NH_3 (Xin et al., 2011). Ammonia is the major noxious gas produced from microbial decomposition of nitrogenous compounds, especially from uric acid (Veens et al., 2009). A chemical method to mitigate NH_3 emissions from litter is the Poultry Litter Treatment (PLT®, sodium bisulfate; Jones-Hamilton CO., Ohio, USA). This is a litter amendment that produces hydrogen ions (H+) by dissolving when applied to the soiled litter substrates. Hydrogen ion

then reacts with NH_3 to form NH_4^+ and the amount of NH_3 emission will be reduced (Li et al., 2006). Although the application of PLT® is considered safe for birds (Li et al., 2013), it has never been investigated whether birds are motivated to access litter substrate treated with PLT®.

Motivation to get access to a specific resource/environment can be tested by assessing how hard an animal is willing to work for a resource by imposing a cost on gaining this resource (Dawkins, 1983). The cost (operant tasks) to get access to a resource can take different forms in birds such as pecking a key (Harlander-Matauschek et al., 2006), squeezing through narrow gaps (Cooper and Appleby, 1996) or pushing doors (Widowski and Duncan, 2000; de Jong et al., 2007). Of these different operandi, weighted push-doors are considered a more naturalistic task for birds to get access to bedding substrate (Cooper and Albentosa, 2003). Generally, animals will work hard for important resources (such as feed) in the face of increasing the costs (such as pushing heavier doors) but will cease to push heavier doors for less important resources (Dawkins, 1983; Petherick and Rutter, 1990). The highest cost (i.e., the heaviest weight pushed, the maximal price paid) indicates motivational strength (Cooper and Mason, 2000).

This study was carried out to assess hens' motivation to access different litter substrates by comparing this with their motivation to access feed (positive control). Hens had to pay a cost (pushing a weighted door) to access one of four litter substrates including soiled wood-shavings, soiled wood-shavings treated with PLT®, unsoiled wood-shavings, and no litter substrate (rubber mats on a concrete floor; negative control). Access to feed was considered the gold standard as hens are highly motivated to feed (Nicol, 2015). The effect of a cost

(pushing a weighted door) on the latency to the first visit, the number of visits, and time spent by hens in different litter substrates and feed treatment were recorded. It was predicted that hens would be willing to push the heaviest weight to gain access to the feed. Based on the findings of previous studies that animals avoid excreta or selectively forage away from excreta (Kavaliers et al., 1997; Cooper et al., 2000), it was hypothesized that the maximum price paid for pushing weighted doors to access litter substrates would show a decreasing trend in the order of unsoiled wood-shavings, soiled wood-shavings, soiled wood-shavings treated with PLT®, and no litter substrate.

5.3 Materials and methods

5.3.1 Ethical statement

The study was approved by the University of Guelph Animal Care Committee (AUP#3169). While planning and conducting the experiment, ARRIVE guidelines were followed (Kilkenny et al., 2010). To promote both refinement and reduction of bird numbers, different genetic lines shared an experimental unit/pen (Walker et al., 2016).

5.3.2 Animals

A total of 84 non-beak trimmed laying hens of four strains (Lohmann Brown, Lohmann LSL Lite, Dekalb White, and Hyline Brown) obtained from the University of Guelph Arkell Research Station at 50 weeks of age, were used for the study. Before this experiment, birds were kept on litter substrate (a mixture of excreta, wood-shavings as bedding material, waste feed, and pieces of feathers) in aviaries as described in Kozak et al. (2016). Hens were kept in six mixed-strain groups with the same number of brown- and white-feathered hens in each group.

5.3.3 Housing and test pen set up

Hens were housed in the experimental room containing six pens (275 cm × 122 cm; 14 hens/pen). A wooden sheet was placed between the width of the two pens, to prevent the transfer of visual information between pens and treatments. There was no provision to remove the olfactory or auditory mode of communication among hens between pens. Each pen (Figure 5.1) was partitioned into two identical compartments: a home compartment (H) and a treatment (T) compartment. Each compartment (137.5 cm × 122 cm) contained five identical nipple drinkers, 9.8 cm of feeder space per hen, two 120 cm long wooden perches (one 50 cm and another 76 cm above the ground) and three metal nest boxes. Black rubber mats covered the floor. Feed and water were provided *ad libitum*. Standard commercial lighting and temperature protocols were followed throughout the experimental period.

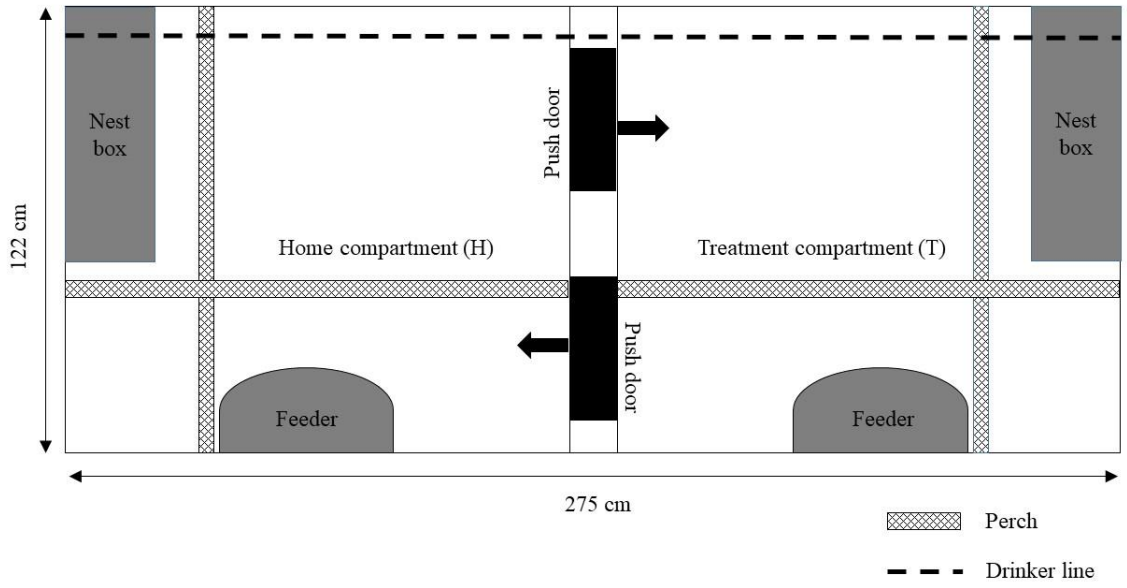

Figure 5.1. Schematic representation of the experimental pen (aerial view, not to scale)

The H compartment was connected to the T compartment (Figure 5.1) by two one-way vertically swinging weighted push doors, each weighing 260 g without door weights. From the H compartment, hens could enter the T compartment through the weighted push door and return to the home compartment through an un-weighted exit door. The push doors used were made of transparent polycarbonate plastic with an opening that allowed the head to fit through to the push door (Figure 5.2). The transparent push door material and the opening allowed a clear view of the substrate behind the closed door. Hollow pipes attached on either side of the push door allowed the addition of lead scale calibration weights. Push doors that were unweighted could be opened by birds using light force at shoulder height (approximately 15 cm). The dimensions of the push door were 41 cm × 33 cm (h × w). Axis height was 41 cm.

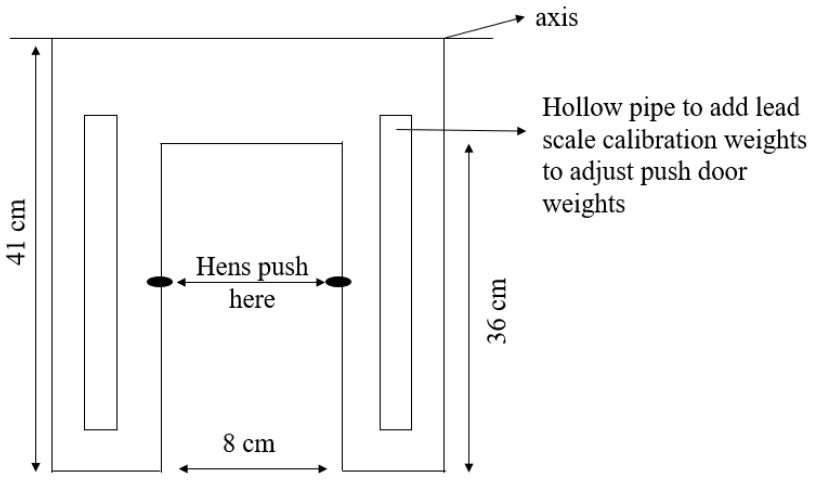

Figure 5.2. Dimensions of push door (not to scale)

The location of the H and T compartment in the test pen systematically alternated. The H compartment (Table 5.1) always contained wood-shavings soiled with excreta/feather parts (built-up over 50 weeks, approximately five cm thick). The T compartment (Table 5.1) contained one of the following substrates: S - soiled wood-shavings; PLT® - soiled wood-shavings treated with a litter acidifier [PLT® - Poultry Litter Treatment, Jones-Hamilton CO., US]; US - unsoiled clean, wood shavings (Pine and Spruce shavings – Pestell Pet Products, Ontario, Canada); NS - no substrate on black rubber mats. The motivation for feed was used as an effective benchmark against which to compare the motivation for the various substrates. This treatment was referred to F, while the feeder in the home compartment was empty. PLT® was spread on the top of the litter as a top dressing 16 hours before use at an application rate of 0.37 kg/m^2 as recommended by the manufacturer (Jones-Hamilton CO., US).

Table 5.1. Summary of treatment combinations used for the experiment showing litter substrates used and availability of feed in different compartments

Treatments[1]	Substrates on the floor[2]		Feed availability	
	H	T	H	T
S	soiled wood-shavings	soiled wood-shavings	yes	yes
PLT®	soiled wood-shavings	soiled wood-shavings treated with PLT®	yes	yes
US	soiled wood-shavings	unsoiled wood-shavings	yes	yes
NS	soiled wood-shavings	no substrate on black rubber mats	yes	yes
F	soiled wood-shavings	soiled wood-shavings	no	yes

[1]S = soiled wood-shavings, PLT® = soiled wood-shavings treated with PLT®, US = unsoiled wood-shavings, NS = no substrate on black rubber mats, F=Feed treatment
[2]H = home compartment, T = treatment compartment

5.3.4 Acclimatization, training and test protocol

The laying hens were allowed to acclimatize to the experimental setup for two weeks. All treatment substrates were introduced and locations of the different substrates in the pen were systematically changed before testing. During the first seven days, the uni-directional push doors remained open so that hens could explore both H and T compartments. The next seven days involved closing the unweighted push doors.

During testing, the H compartment with soiled wood-shavings acted as a starting point of the test in each pen. Whereas, at each testing cycle, the T compartment varied between pens. The treatment combination for each pen was changed after each testing cycle (eight days). This means that a pen receiving a particular treatment combination was changed to another treatment combination after each testing cycle systematically (For example, S-US combination during one testing cycle could be changed to S-PLT® combination during the next testing cycle and so forth). The treatment combination was changed in such a way that each pen received all the treatment combination during the study. During each testing cycle, four different weights were added to the push door (towards the T compartment) every 23 hours (one hour was allocated to change weights of the push door). Push door weight was not gradually increased, instead, systematically varied to minimize order effects. The following weights were added to the doors: 0% of average body weight of hens; 500 g (20% of the average body weight of the hens per group; 1100 g (40% of average body weight of hens); and 1500 g (60% of average body weight of hens)]. The push door weights used for the study were calculated using the average weight of a hen per group taken at the start of the experiment. After completion of four days of testing using four different weights in each pen, the whole procedure was repeated for the next four days (total of eight days per testing cycle). The location of the weighted and unweighted doors was also randomly changed after the completion of each testing cycle of testing per pen. The exit door (T to H compartment) remained unweighted throughout the experiment.

5.3.5 Litter sampling and analysis

Litter samples were collected at the start of each testing cycle. Samples were collected from three locations inside the pen and combined to create a homogenized grab sample representative of the entire pen. Samples were sent to Agri-Food Laboratories - SGS (Guelph, Canada) to analyze moisture (%), pH, total nitrogen (N) (%), and ammonia nitrogen (NH_3-N) (ppm). The results of the laboratory analysis of litter samples are shown in Table 5.2.

Table 5.2. Results of laboratory analysis of different litter substrates

Treatments	Moisture (%)	pH	Total nitrogen (%)	Ammonia nitrogen
S	19.79 ± 2.818	7.65 ± 0.422	3.71 ± 0.194	2986.58 ± 581.140
US	17.74 ± 2.582	6.26 ± 0.599	0.53 ± 0.070	405.16 ± 177.702
PLT®	18.09 ± 3.870	6.81 ± 0.743	3.75 ± 0.057	2868.16 ± 481.518

5.3.6 Bird observation

Individual hens were tracked using radio-frequency identification (RFID) technology (Biomark®, Boise, Idaho, US). All birds were fitted with an adjustable plastic leg band (medium-sized brown – Roxan®, UK) to fit a passive integrated transponder (Biomark® HPT 12 PIT tag, 12.5 mm, 134.2 kHz). Using a handheld PIT tag reader (Biomark®), a unique PIT tag was assigned to each hen to track the location. Below each push door, an antenna (30 cm × 10 cm × 2 cm) (Biomark®, Boise, Idaho, US) was placed that read PIT tags fitted inside the leg band while hens pushed the door. When a hen with a PIT tag stepped into another compartment, the PIT tag was activated by the radio frequency field generated by

the antenna placed under the push door. This information was transmitted to the antenna, which was then recorded on a microchip. The information on the microchip was transferred to a computer using a microchip reader. The power supply to the antenna was provided by MW 122 power supply including 3-prong cable kept inside the portable enclosure (as recommended by the manufacturer). To prevent reading of multiple PIT tags entering towards the same direction, the antenna was placed in such a way that only one hen entering toward the T compartment could step on the antenna at one time. However, there was a potential of reading of PIT tags of the hens already present in the other compartment causing false positives in the dataset. Such false-positive data were removed later on during the data analysis. The number of visits and duration in each compartment was computed for each 23 h period (one hour out of 24 hours was allocated to change weights of the push door).

5.3.7 Behavioural measurement

On each test day, hens started in the H compartment (10:00h) and the location and the total time spent in H and T compartments (S, US, PLT®, NS and F) was recorded over a period of 23 hours. The following parameters were calculated from the PIT tag observations of each hen in the experiment: the latency in hours (how long each hen takes to enter into T compartment), the number of visits to the T compartment and the time spent in hours in various substrates at T compartment. The maximum price paid (maximum door weight pushed) by hens (in g) to access various substrates in the T compartment was also calculated.

5.3.8 Statistical analysis

Data obtained were analyzed using a PROC GLIMMIX procedure of the SAS (v9.4, SAS Institute., Cary, NC) statistical software package. Response variables used were (1) maximum price paid (maximum door weight pushed), (2) latency to enter T compartment (hours), (3) number of visits (counts), and (4) time spent (hours) in the T compartment. Treatments (S, US, PLT®, NS and F), weight of the push door (0, 20, 40 or 60% of average body weight per group), strain (brown-feathered hens and white-feathered hens) and their three-way interaction were included as fixed effects in the model, and random statement included sequence of the weight added on the push door and bird within pen as an experimental unit. As significant differences ($P < 0.05$) were observed between brown- and white-feathered birds for most of the parameters measured except maximum price paid, separate ANOVAs were performed for each strain. For the analysis of maximum price paid, treatments, strain and their two-way interaction were included as fixed effects in the model. A Tukey-Kramer adjustment was used for multiple means comparisons. The maximum price paid in g, latency and the number of visits was fit with a log link to achieve normality and random distribution of residuals. Means and standard errors on the data scale were obtained using the Glimmix ilink option. Models analyzing variation in the time spent (hours) in the T compartment had a Gaussian distribution with an identity link function. Due to the repeated measures taken on the same group of birds (pen) at different time points, an autoregressive covariance structure of order 1 was fitted. The degrees of freedoms were adjusted using the Kenward–Roger method. All results are presented as least square means (LSM) ± standard error of the mean (SEM). Statistical significance was considered at $P < 0.05$ in all cases.

5.4 Results

5.4.1 Motivation to access substrates

The maximum price (push doors with variable weights) paid by laying hens to access substrates differed between NS and all other treatments ($F_{4, 250.4}$ = 15.41; P < 0.0001). Laying hens were similarly motivated to access treatments US, S, PLT® and F (Figure 5.3) but were more motivated (P < 0.0001) to access these resources than treatment NS. No differences ($F_{1, 119.3}$ = 2.66; P = 0.105) were found between brown- and white-feathered strains in the maximum weights pushed to access various substrates.

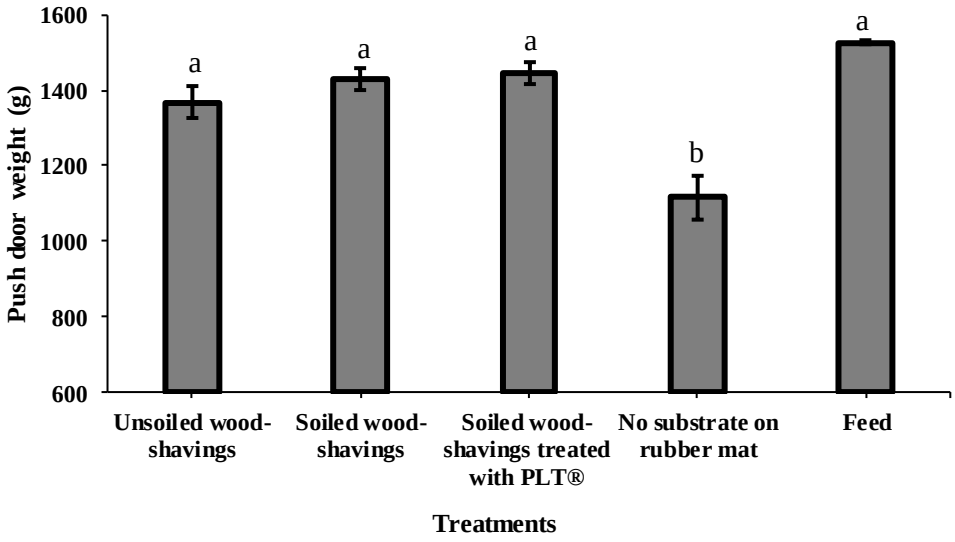

Figure 5.3. The maximum price paid (g) to access the different treatments/substrates by hens. Different letters (a and b) represent treatments that differed significantly (P < 0.05).

5.4.2 Latency to access various substrates in the T compartment

The time taken by hens to access various substrates (descriptive results) in the T compartment is shown in Table 5.3. There was a significant interaction between push door

weight and litter treatments for brown- ($F_{12, 883.1} = 1.78$, $P = 0.046$) and white- ($F_{12, 955} = 4.76$, $P < 0.0001$) feathered hens. Latency to enter to the treatment F was lowest at all push door weights for both white- and brown-feathered hens (Figure 5.4). There were no consistent significant differences among four different litter substrates (US, PLT®, S and NS) at four different push door weights (Figure 5.4); however, in general, the latency was in the order of US, S, PLT® and NS from the lowest to the highest for brown-feathered hens and in the order of US, PLT®, S, and NS from the lowest to the highest for white-feathered hens.

Table 5.3. Time taken (hours) by brown- and white-feathered hens to access various substrates in the T compartment

Item		Strain	
		Brown-feathered	White-feathered
Treatments[1]	F	3.37 ± 0.419	2.76 ± 0.345
	US	6.94 ± 0.850	10.72 ± 1.273
	PLT®	8.94 ± 1.082	11.65 ± 1.403
	S	8.55 ± 1.325	12.60 ± 1.498
	NS	14.97 ± 1.082	16.91 ± 2.009

[1]F=Feed treatment, US = unsoiled wood-shavings, PLT® = soiled wood-shavings treated with PLT®, S = soiled wood-shavings, NS = no substrate on black rubber mats

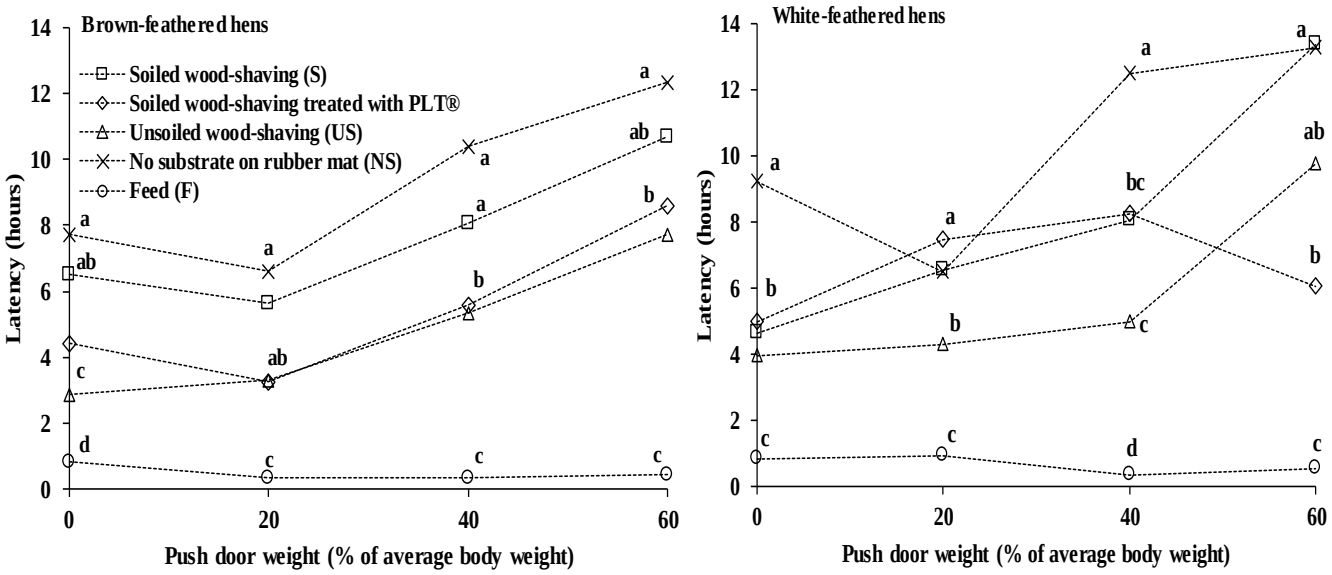

Figure 5.4. The effect of door weight on the latency (hours) to access various substrate treatments in the T compartment. Different letters (a, b, c and d) represent treatments that differed significantly ($P < 0.05$) for a given push door weight.

5.4.3 Number of visits to different treatments in the T compartment

The average number of visits by hens to various substrates (descriptive results) in the T compartment during the 23 hours test period is shown in Table 5.4. The number of visits was found to be very low (ranging from 0.94 ± 0.142 to 3.54 ± 0.295) to the T compartment for a period of 23 hours. There was a significant interaction between push door weight and litter treatments for brown- ($F_{12, 696.5} = 2.44$, $P = 0.004$) but not for white- ($F_{12, 302.7} = 0.99$, $P = 0.4588$) feathered hens. There was no significant difference ($P > 0.05$) in the number of visits to S, NS, and PLT® at most of the push door weights (Figure 5.5). For white-feathered hens, the number of visits to treatment NS was found to be lowest at all push door weights (Figure 5.5).

Table 5.4. Number of visits by brown- and white-feathered hens (per 23 hours) to different treatments

Item		Strain	
		Brown-feathered	White-feathered hens
Treatments[1]	F	3.54 ± 0.295	3.20 ± 0.285
	US	3.08 ± 0.272	2.15 ± 0.222
	PLT®	2.61 ± 0.252	2.12 ± 0.228
	S	2.42 ± 0.230	2.12 ± 0.222
	NS	1.54 ± 0.190	0.94 ± 0.142

[1]F=Feed treatment, US = unsoiled wood-shavings, PLT® = soiled wood-shavings treated with PLT®, S = soiled wood-shavings, NS = no substrate on black rubber mats

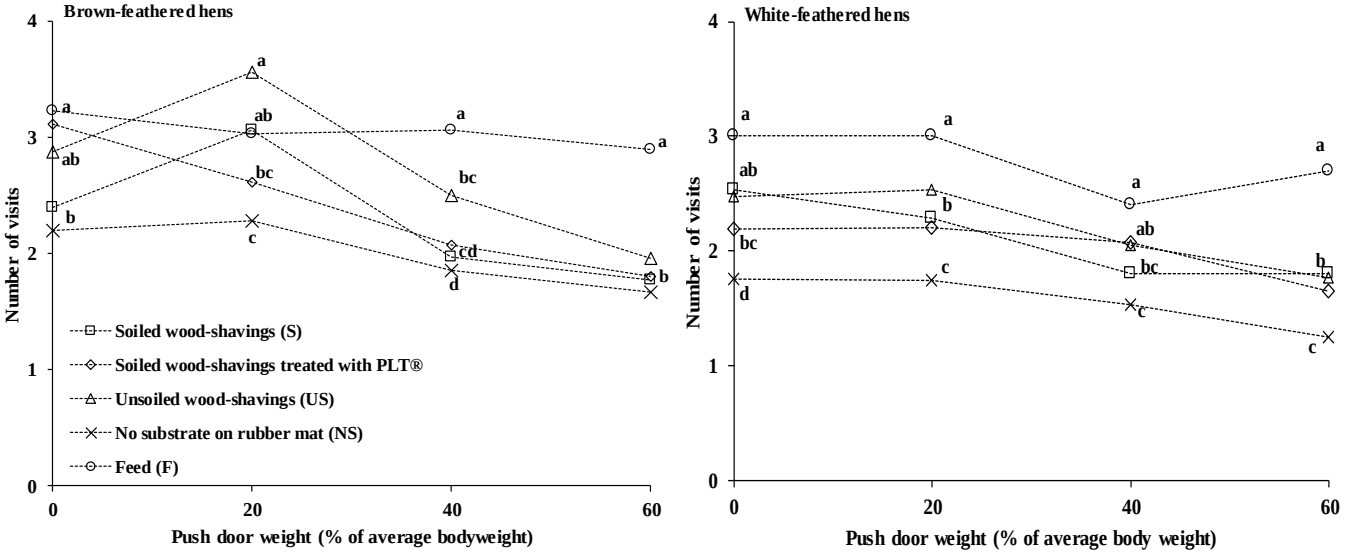

Figure 5.5. The effects of door weight on the number of visits per 23 hours to access various substrate treatments in the T compartment. Different letters (a, b, c, and d) represent treatments that differed significantly ($P < 0.05$) for a given push door weight.

5.4.4 Time spent in different treatments in the T compartment

Table 5.5. shows the time spent by hens in T compartment (descriptive results) for the different treatments for the duration of 23 hours testing period. Numerically, time spent in different treatments was in the order of F, S, US, PLT® and NS from the highest to the lowest for brown-feathered hens whereas, in the order F, S, PLT®, US, and NS from the highest to the lowest for white-feathered hens. There was a significant interaction between push door weight and litter treatments for brown- ($F_{12, 696.5} = 6.76$, $P < 0.001$) and white- ($F_{12, 897} = 5.06$, $P < 0.0001$) feathered hens. Hens spent significantly less time in NS at all push door weights (Figure 5.6). However, there was no consistent significant difference in time spent among other treatments (Figure 5.6).

Table 5.5. Time spent (hours) by brown- and white-feathered hens in different substrate treatments

Item		Strain	
		Brown-feathered	White-feathered
Treatments[1]	F	10.99 ± 0.732	9.65 ± 0.730
	US	6.95 ± 0.731	5.01 ± 0.719
	PLT®	6.83 ± 0.735	6.38 ± 0.721
	S	8.79 ± 0.731	8.34 ± 0.713
	NS	3.23 ± 0.733	1.37 ± 0.714

[1]F=Feed treatment, US = unsoiled wood shavings, PLT® = soiled wood shavings treated with PLT®, S = soiled wood shavings, NS = no substrates on black rubber mats

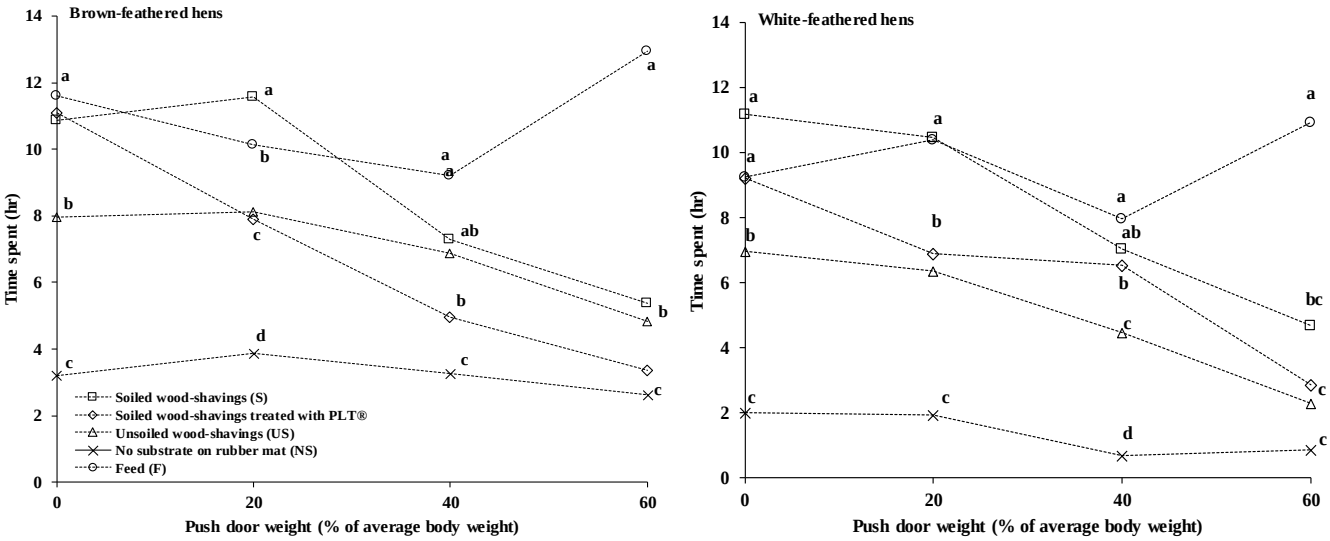

Figure 5.6. The effects of door weight on time spent (hours) out of 23 hours in various substrate treatments at the T compartment. Different letters (a, b, c, and d) represent treatments that differed significantly ($P < 0.05$) for a given push door weight.

5.5 Discussion

This study investigated hens' motivation to access different floor litter substrates (S, US, PLT®, and NS) by comparing this with their motivation to access feed (F) (positive control). Contrary to the predictions, laying hens were equally motivated to access different litter substrates (S, PLT® or US). Analysis of maximum price paid, latency, the number of visits and visit duration did not demonstrate a particular preference for any type of substrate (S, PLT® or US) in hens.

In this study, hens did not avoid soiled wood-shavings on the floor as hens were observed spending considerable time out of their time budget in soiled wood-shavings in both H and T compartments. Studies in domestic animals have reported that many domestic animals forage away from excreta or excreta-soiled area (Forbes and Hodgson, 1985, Cooper et al., 2000). However, Von Waldburg-Zeil et al. (2018) reported excreta consumption in laying hens. Additionally, Pokharel et al. (2018) also observed laying hens foraging on excreta-soiled scratch pads. This could explain why laying hens did not avoid soiled wood-shavings in this study.

Additionally, in this study, hens were as motivated to enter a compartment containing PLT® treated litter as those containing soiled or unsoiled litter substrates. Use of PLT® ($NaHSO_4$) effectively reduces litter pH, reduces NH_3 volatilization and inhibits microbial activity (Jones-Hamilton, 2019), thereby reducing the incidence of pododermatitis (Nagaraj et al., 2007). Interestingly, in this study, the extent to which PLT® reduced litter pH was smaller than in the study conducted by Wood (2015) in a commercial turkey facility. Shah et al. (2012) indicated that PLT® needs sufficient moisture to be activated. The lower litter moisture (19%) in this study than the study by Wood (2015) (42%) might be the reason why pH was not reduced as expected. Furthermore, Wood (2015) found the drop in litter pH after PLT® application to be very short-lived. This minimal effect on litter pH and NH_3 could be why hens displayed an equal motivation to access soiled wood-shavings and PLT® treated wood-shavings, as hens might not have differentiated these two litter substrates.

Hens preferred floor substrate (bedding) over no substrate, which is consistent with the findings of previous studies (Dawkins, 1983; Matthews et al., 1995). In addition, analysis of

the maximum price paid did not reveal a difference between US, S, PLT® and feed (F). Laying hens use floor litter substrates extensively for pecking and scratching, dustbathing, nesting and resting (Cooper and Albentosa, 2003; Hartcher et al., 2015; Campbell et al., 2017). Van Staaveren et al. (2018) reported that over 20% of non-cage laying hen housing systems in Canada did not provide bedding materials on the floor. Therefore, based on our findings, litter substrates should be considered a minimal requirement when housing hens in non-cage systems. Furthermore, the Canadian Code of Practice for the Care and Handling of Pullets and Laying hens (NFACC, 2017) requires that "hens housed in litter-based systems must be provided with continuous access to litter".

Hens' motivation to access substrates could be attributed to experience with floor litter substrates. In this study, hens were reared on built-up litter substrate for life and did not have experience on non-littered flooring. Experience with a resource can affect hens' choices (Petherick et al., 1990; Nicol et al., 2001). Additionally, social interaction may influence hens' decisions as they started at the same location (Nicol et al., 1999; Weeks and Nicol, 2006).

During the 23-hour testing period, the number of visits and visit durations to the weighted T compartment was very low. Hens may have flocked together in the H compartment resulting in fewer visits to the T compartment. Additionally, apart from foraging on litter substrates, hens may have spent a substantial amount of time in non-littered areas of the pens (e.g. perches and nest boxes). Nevertheless, the hens' few visits to the T compartment suggests that they did not avoid the soiled litter substrate in the H compartment.

Although the hens were habituated to the different types of floor litter substrates, the novelty of the litter substrates present in the treatment compartments could affect their decisions. Although the use of novel objects (litter substrates in this study) helps reduce fear responses in the long run (Newberry, 1999), there is potential for an early fear response when new objects are added (Jones and Carmichael, 1999). This might also be one of the reasons for hens' limited movement towards the treatments. Additionally, this might also explain why white-feathered hens had a lower number of visits to the treatment compartments and took longer to enter the treatment compartments compared to brown-feathered hens. Studies suggest that white-feathered hens are generally more fearful than brown-feathered hens (de Haas et al., 2013; de Haas et al., 2017). Fearfulness and tendency to escape from dominant hens could also influence these results. For the most fearful hens in the group, two alarming situations may have happened concurrently: dominant hens in the home compartment and novel objects in the treatment compartment. Therefore, those hens might have chosen to stay on the perches, which could also affect these results.

Usually, motivation tests are conducted in isolation, as an animal tested in a group may perform an operant task to gain social interaction rather than to gain the resources provided (Sherwin, 2003; Kirkden and Pajor, 2006). Further, determining whether or not the responses of individuals are attributed to the group may be difficult while testing in groups. Hens are highly social animals; therefore, social context should be considered while analysing the results (Pedersen et al., 2002). Hens may choose to stay with or search for conspecifics, which could confound the results of consumer demand studies conducted in

groups (Pedersen et al., 2002). Therefore, it is recommended to conduct these studies both in isolation and in groups to analyze both aspects.

There were some limitations in the study. Due to construction limitations, push doors did not have weights more than 60% of the hens' body weight. Push door weights heavier than 60% of the hens' bodyweight would provide deeper insight into the maximum price paid. Additionally, the small number of visits to the treatment compartment during the 23-hour testing period might also have affected some of the parameters presented. In the study, the price imposed on the push door was systematic. Therefore, without a gradual increase in push door weight, hens pushing the heaviest weight during early testing could decide not to push the door later in the experiment. While analyzing these results, the data from white- and brown- feathered hens were treated separately (except for maximum weight pushed which found no difference between brown- and white-feathered hens). Future studies should consider the difference in weight of individual birds as well the impact of social interaction on the outcome of push door studies.

In summary, these results show that adult laying hens are equally motivated to access unsoiled, soiled, and PLT® treated soiled litter substrates, as well as feed. Also, hens are more motivated to access floor litter substrates compared to the floor with no litter substrate. The importance of litter substrates to laying hens indicates that laying hen farmers should prioritize adding litter substrate when housing them. Further study is needed to understand how long-term exposure to excreta-soiled litter affects laying hens.

5.6 Acknowledgements

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Chapter 6

6 Effect of low-protein energy-rich diet on body weight, plasma hepatic markers, hepatic damage, and learning of adult laying hens

6.1 Abstract

Although feeding low-protein energy-rich diets contribute towards reducing environmental pollution, it can increase the susceptibility to liver damage in laying hens. Liver damage in animals has been associated to impair their learning behavior. The information regarding the impact of low-protein energy-rich diets on birds' learning ability is limited. This study aims to understand the impact of low-protein and energy-rich diets on body weight, hepatic markers and learning abilities in hens. Adult laying hens (brown- and white-feathered birds) received three dietary treatments with different crude protein and energy levels [Control diet (18% CP and 2900 Kcal/kg ME), and two lower protein diets with 2% less CP (16% CP and 3000 Kcal/kg ME, herein referred as 16% CP diet) and 6% less CP (12% CP and 2800 Kcal/kg ME, herein referred as 12% CP diet)] for a total of 16 weeks. There was no dietary impact on body weight, plasma hepatic markers indicating liver damage or discrimination reversal learning abilities in hens. Interestingly, brown-feathered hens learned faster than white-feathered hens. Also, hens with a higher level of plasma alkaline phosphatase (ALP) were less successful in learning compared to those with a lower level of plasma ALP. This result is consistent with the findings that elevated plasma ALP level is inversely correlated with cognitive function in mammals. These findings demonstrate that environmentally friendly lower dietary protein levels (up to 12% CP) do not impact liver health and learning of adult laying hens.

Keywords: laying hens, low-protein energy-rich diet, hepatic damage, cognition, behaviour.

6.2 Introduction

Indoor and ambient NH_3 and dust can reach unnaturally high concentrations in poultry facilities that can cause various health and welfare problems in birds and human workers (Wathes et al., 2002; Nicholson et al., 2004; Wood and Van Heyst, 2016). Ammonia emissions from poultry litter are the result of microbial degradation of nitrogenous compounds, such as undigested proteins and uric acid, in poultry excreta (Wood and Van Heyst, 2016). To reduce NH_3 release from chicken excreta, some farmers choose to decrease the protein content, of their hens' diet resulting in an imbalance of energy to protein ratio (Nahm, 2007; Roberts et al., 2007a; Roberts et al., 2007b). While this may be a method of improving the air quality and reducing the ecological footprint, the possible consequences of long-term consumption of low protein diets on bird's health and welfare have not been fully investigated.

Low-protein energy-rich diets (LPER) can contribute to hepatic metabolic disorders, such as fatty liver hemorrhagic syndrome (FLHS) in caged and backyard laying hens (Leeson, 2007; Jiang et al., 2013; Trott et al., 2014; Rozenboim et al., 2016; Robinson and Kiarie, 2019). Fatty liver hemorrhagic syndrome, a leading non-infectious cause of mortality in laying hens due to hepatic rupture and hemorrhage (Mete et al., 2013; Shini, 2014), is characterized by the accumulation of fat deposited in the liver (Trott et al., 2014). As excessive fat accumulates in the liver, liver capsules can rupture leading to hemorrhage (Shini, 2014). Resulting compromised liver function is manifested by a variety of metabolic disturbances and variable clinical and hematological signs such as increased hepatic plasma enzyme activities (Lumeij, 1994). Although other factors such as ambient

temperature or bird genetics can contribute to the development of hepatic metabolic disorders, nutritional imbalances such as low-protein and high-energy levels appear to be critical (Leeson, 2007; Rozenboim et al., 2016).

Excessive consumption of high-energy processed food has been identified as the main cause of metabolic disorders (e.g., obesity) co-occurring with non-alcoholic fatty liver disease in humans (Larter et al., 2010; Mager et al., 2010). Interestingly, these metabolic disorders can lead to negative health implications including cognitive dysfunction (Spencer et al., 2017). Moreover, non-alcoholic fatty liver diseases in humans are associated with inferior learning and recall (Seo et al., 2016). Although the pathobiology behind this is unclear, the accumulation of ammonia (in gaseous (NH_3) and ionic (NH_4^+) forms) in the blood and in the brain can reach neurotoxic concentrations, due to its limited metabolism to urea and uric acid by the diseased liver (Toris et al., 2011; Skowronska and Albrecht, 2012). This may partially explain cognitive/learning impairments, due to the disturbances in brain neurotransmission, cellular energy metabolism, and cell swelling from severely diseased livers (Skowronska and Albrecht, 2012). Severe hepatic damage in animal (rodents) models also produces hyperammonaemia and has been seen to impair a variety of behavioural/cognitive tasks (Bengtsson et al., 1986; Apelqvist et al., 1999; Aguilar et al., 2000) including cognition/visual discrimination reversal learning (Méndez et al., 2010).

There have been several studies addressing the effects of LPER diets on visual discrimination and/or visual discrimination reversal learning in humans and other mammals showing that they performed more poorly on learning reversal problem tasks (measuring the flexibility of a response), compared to mammals fed a standard diet (Zimmermann, 1973;

O'Connel et al., 1978; Reyes-Castro et al., 2011). Interestingly, even though domestic birds are farmed worldwide in both large and small scale (Appleby et al., 2004), there is a general lack of information on how dietary protein affects liver health and cognition in these birds. A recent study found that broilers across all dietary protein treatments had a high incidence of liver damage that did not become worse when feeding LPER diets and did not impact visual reversal learning (Bona et al., 2018). However, limited information is available on laying hens. Therefore, the objectives of the present research were twofold. First, it was hypothesized that LPER diets would decrease body weight, and increase the risk of hepatic damage (indicated by plasma hepatic markers and plasma NH_3 levels). Second, it was hypothesized that hens on LPER diets would show impaired reversal learning on a motor and visual two-choice discrimination task. Thus, hens with the highest levels of NH_3 and plasma liver enzymes were expected to perform worse on the behavioural tasks, which could be impacted by genetic strain (Albentosa et al., 2003; Fraisse and Cockrem, 2006; de Haas et al., 2013).

6.3 Materials and methods

6.3.1 Ethical statement

The research protocol was approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol # 3169) before the start of data collection. While planning and conducting the experiments, ARRIVE guidelines were followed (Kilkenny et al., 2010).

6.3.2 Animals, housing and dietary treatments

For this experiment, a total of 73 laying hens (72 weeks of age) were used. Hens were housed in 6 identical pens in a mixed-strain group (Lohmann Brown, Hyline Brown, LSL

Lite, and Dekalb White) to prevent disrupting their already established social structure and to avoid the stress that would impact their learning behaviour. Each pen (275 cm × 122 cm) was equipped with 9.8 cm of feeder space per hen, 10 nipple drinkers, wood-shavings as a litter substrate, two perches (one 100 cm long mounted 50 cm above the ground and another 120 cm mounted 76 cm above the ground), and three nest boxes. An opaque whiteboard was placed in between pens to avoid visual contact between birds of adjacent pens, which might lead to social learning. Standard commercial lighting and temperature protocols were followed throughout the experimental period.

A timeline of the experiment is depicted in Figure 6.1. Before the start of the dietary treatments and during baseline behavioural testing, all hens were maintained on the same 18% crude protein (18% CP 2,900 Kcal/kg ME) diet supplied by Arkell Poultry Research Station. After baseline behavioural testing (5 weeks in duration), pens of birds were assigned to three dietary treatments with different crude proteins and different ME levels [Control diet (18% CP and 2,900 Kcal/kg ME; n = 26) – the same diet supplied by Arkell Poultry Research Stations, and two lower protein diets with 16% CP (16% CP and 2,800 Kcal/kg ME; n = 21) and 12% CP (12% CP and 3,000 Kcal/kg ME; n = 26) over a period of 16 weeks (Table 6.1). The three diets were corn and soybean meal-based diet and formulated to meet the nutrient requirement of hens at 72 weeks of age except 12% CP diet that was formulated to created imbalance in energy to protein ratio. The diets were in crumbled form, prepared with the same batch of ingredients at the Arkell Research Station feed mill. Table 6.1 shows the composition of the diets used in this experiment.

Table 6.1. Ingredient composition and calculated nutrient composition of three experimental diets[1]

Ingredients, %	Treatments		
	Control (18% CP)	16% CP diet	12% CP diet
Corn	55.50	56.34	62.00
Wheat	5.10	2.40	2.50
Soybean meal	16.60	23.64	14.45
Pork meal	7.00	-	-
Corn gluten	2.50	-	-
Ani-veg fat	2.50	5.70	8.80
Limestone	9.50	9.00	9.00
Monocal-P	0.55	1.30	1.40
Common salt	0.30	0.35	0.35
Vit-Min premix	-	1.00	1.00
RAC vit. Booster	0.15	-	-
RAC broiler micro	0.15	-	-
Lysine	-	0.10	0.30
Methionine	0.10	0.17	0.20
Choline Chloride	0.05	-	-
Calculated provisions,			
ME, Kcal/kg	2900	2800	3000
CP	18.00	16.01	12.30
Ca	4.20	3.52	3.51
avP	0.40	0.38	0.38

Lysine	0.89	0.70	0.70
Methionine	0.38	0.36	0.36
Sodium	0.18	0.16	0.16

[1]Provided with the following quantities of vitamins and minerals per kg of a vitamin premix: Vitamins: A, 8800 IU; D3, 3300 IU; E, 40 IU; B12, 12 mcg; Biotin, 220 mcg; Thiamin, 4 mg; vitamin B2, 8 mg; pantothenic acid, 15 mg; niacin, 50 mg; folic acid, 1 mg; copper, 10 mg; iron, 60 mg; choline, 600 mg.

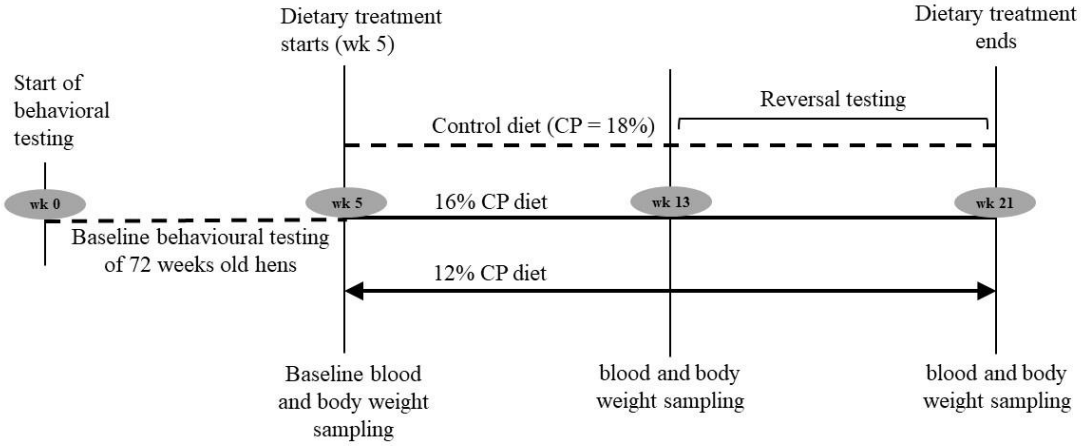

Figure 6.1. Timeline detailing points of behavioural and metabolic testing. All birds received the same Control (18% CP, ME) diet during baseline behavioural testing (35 days). After baseline behavioural testing (week 5), birds received either the Control diet (dashed line), 16% CP diet (solid line) or 12% CP diet (arrowed line) for the remaining 16 wks of the study. At the end of week 13, reversal learning testing commenced. Blood and body weight sampling was conducted in week 5, week 13, and week 21. Reversal learning testing occurred between weeks 13-21.

6.3.3 Body weight, blood sampling, and analysis

The hens were individually weighed at the start and at weeks 5, 13 and 21 of the experiment. At the start of the dietary treatment and at 8 and 16 weeks of the dietary treatment, blood samples were drawn from the wing vein of each bird between 10:00 and 10:30 am over a period of two days involving two blood collectors. Upon collection of 2 mL of venous blood

using 22-gauge needles into lithium heparin tubes, samples were rapidly transported to the laboratory and centrifuged for plasma separation (3,000 × *g* at 4°C for 10 min). For the analysis of plasma NH_3, fresh plasma was deproteinized within one hour of blood collection, and a colorimetric reaction was performed within 24 hours of deproteinization according to the protocols of McCullough (1967). High blood NH_3 levels may indicate reduced liver function and/or NH_3 poisoning (Lumeij, 1994; Olde Damink et al., 2002; Harr, 2006).

Plasma samples were stored in aliquots at -20^{o} C for the analysis of plasma enzymes indicating cellular liver damage. Analysis of plasma enzyme profiles included aspartate aminotransferase (AST), which is an indicator of avian liver and muscle damage (Harr, 2006), gamma-glutamyl transferase (GGT), which indicates compromised hepatic and biliary function in birds (Lumeij, 1994; Harr, 2006), and alkaline phosphatase (ALP), a non-specific indicator of liver and bone disease (Kellett et al., 2011). Plasma AST, GGT, and ALP levels were analyzed using the Roche Cobas C AST kit ID 0-494, Roche Cobas C GGT-2 kit version 2, and Roche Cobas C ALP kit ID 0-495 (Roche Diagnostics, Indianapolis, IN, USA) respectively at the Animal Health Laboratory at the University of Guelph.

6.3.4 Behavioural experiments

Behavioural experiments were performed in a testing area separate from the home pens using protocols employed in a previous study in lizards (Leal and Powell, 2012). Hens were maintained on a restricted food regime for one hour before the start of the behavioural experiment.

6.3.4.1 Testing area and behavioural testing apparatus

The testing area (Figure 6.2) consisted of two compartments: the first compartment, referred to as the start box (46×38×48 cm^3), which opened into a wooden box compartment (61×58×50 cm^3), referred to as the testing arena. A plastic mesh was placed on top of the arena to prevent birds from jumping out of the test area. The start box and the testing arena were separated by a white, plastic door, which the experimenter could open and close by sliding vertically.

For video recordings, a wooden stand was attached to the back of the testing arena. An iPad (model number: A1566, Apple Canada Inc., Toronto, ON) was secured to the wooden stand so that its camera faced down 107 cm above the testing arena, providing an aerial view of the whole testing arena. The iPad was connected, via an HDMI cable, to an external monitor to allow experimenters to view the testing arena without disturbing the hen inside.

A modified Tchibo® cat toy (Tchibo®, Germany) was placed inside the testing area Figure 6.2, in front of the wall opposite to the sliding door. Original Tchibo® had three grooves, which was modified in such a way that only two grooves were visible to experimental hens. Modification of the original toy was conducted by covering the middle groove with blue tape. The modified Tchibo® cat toy was a blue, wooden, circular platform (diameter = 16 cm) containing two grooves. At the end of each of these grooves was a well (diameter = 2.2 cm). To conceal their contents, the wells were covered by circular discs (diameter = 4 cm), which could be easily slid open along the grooves. Each disc was colored either red or green, and food rewards were placed inside one of the wells. Each hen was randomly assigned to a color, either red or green. The position of the rewarded color stimulus (right or left) was

randomly determined before each trial. The reward (equal amounts of feed, raisins, mealworm, and boiled eggs) was placed under the appropriately colored disc, and each hen had to remove the disc of its assigned color to reveal the food reward. To ensure that hens did not use olfactory cues to determine the location of the hidden food reward, equal amounts of the reward were placed under a hidden compartment under each of the wells when wells were covered entirely.

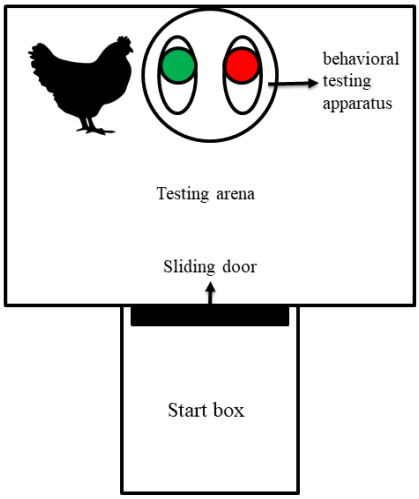

Figure 6.2. Experimental set-up of the testing arena and apparatus (not to scale).

6.3.4.2 Behavioural testing

The timeline of behavioural testing is described in Figure 6.3.

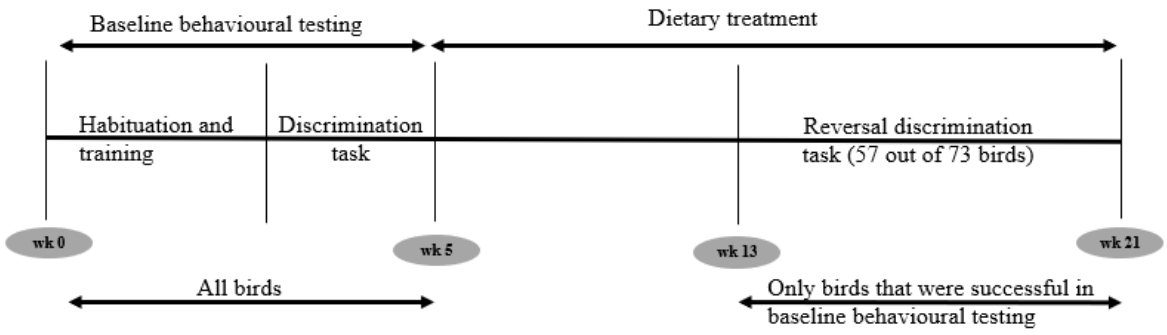

Figure 6.3. Timeline detailing points of behavioural testing. All birds received the same control (18% CP and 2900 Kcal/kg ME) diet during baseline behavioural testing (started at week 0 and ended at week 5). At week 5, birds received dietary treatments over a period of 16 weeks. At the end of week 13, reversal discrimination task commenced.

6.3.4.2.1 Habituation and training

Hens were first habituated to both the testing area and the testing apparatus. They were then trained through a shaping process, which was performed in three sequential stages: (i) both circular discs were positioned next to, but without covering, their respective wells; (ii) both circular discs were positioned covering one-third of their respective wells; (iii) both circular discs were positioned covering two-thirds of their respective wells. In each step, a food reward was placed inside only one well of the hen's previously assigned color. Birds completed a stage and moved to next when they made the correct choice five out of six times.

6.3.4.2.2 Discrimination task

Once the shaping process was completed, both discs, either green (Senaratna et al., 2012) or red (Taylor et al., 1969) colored, were positioned to cover each well entirely. Half of the birds were assigned to the red disc, while the other half was assigned to a green disc pairing an action (dislodging the colored disc) with an outcome (provision of a food reward). This presented birds with the novel motor task (sliding a disc backward in a curved direction) and two-choice visual discrimination task of completely dislodging the disc of their assigned color with their beak or feet to access the food reward. The position of the correctly colored disc (i.e., on the left or right side of the apparatus) and consequently, the food reward, was randomly determined before each trial. After the start box door was opened in each trial, the hen had three seconds to enter the testing area on its own, otherwise, it was gently directed to the testing arena by the experimenter. Individuals conducted one session, consisting of six trials, per day. The choice was scored as the first disc dislodged by the hen. The bird was considered to have successfully learned this task once it made the correct choice on 5 out of 6 trials, on two consecutive days. When no choice was made, or the disc was not dislodged, the trial ended after 30 seconds.

6.3.4.2.3 Discrimination reversal task

Birds that learned [57 out of 73 birds (33 brown-feathered hens and 24 white-feathered hens)] to dislodge the disc performed the discrimination reversal task during dietary treatment testing (Fig 6.3). In the reversal learning task, the same procedure was used as in the previous task; however, the reward contingencies were reversed, i.e., the food reward was now placed in the well covered by the disc of the opposite color (green for red or vice-

versa). This means the trained, previous action/response no longer resulted in a reward, which emphasizes the need for the bird to withhold the initially-trained action/response and, instead, learn that the previously learned response is now futile. Again, hens were considered successful if they were able to dislodge the rewarded disc in 5 out of 6 trials on two consecutive days. Reversal testing was terminated once more than 75% of the birds were successful and completed the task.

6.3.5 Statistical analysis

All statistical procedures were conducted using SAS V9.4 (SAS Inst. Inc., Cary, NC). The model was fitted using PROC GLIMMIX based on the mixed modeling approach for randomized experiments with repeated measures (Piepho et al., 2004). For the analysis, the two brown-feathered (B) and the two white-feathered (W) strains were combined. To analyze the effect of dietary treatment (Control diet, 16% CP diet, and 12% CP diet), strain (White, Brown), weeks (wk 13, wk 21) and their interactions on body weight, plasma liver enzyme levels, and plasma NH_3 levels, a generalized linear mixed model was employed using body weight (wk 5), plasma NH_3 level (wk 5), and plasma liver enzyme activity (wk 5) as covariates in the model. The data was fitted with a negative binomial distribution with a log link and, the random statement included week and bird within pen as an experimental unit. Due to the repeated measures taken on the same hens, an autoregressive covariance structure of order one was fitted, the degrees of freedom were adjusted using the Kenward-Roger method and a Tukey-Kramer multiple comparison adjustment for the p-values for the differences of LS-means were used. The GLIMMIX ilink option was used to convert means and standard errors to the data scale.

To analyze the effect of dietary treatment, strain, and their interaction on the number of sessions required to successfully complete discrimination task and discrimination reversal task, a generalized linear mixed model was used with the fixed effect of dietary treatment, strain, and their interaction, and bird within pen was used as an experimental unit. For each of the behavioural tasks, the number of sessions required to achieve the learning criterion in the previous task was included as a covariate. The data was fitted with a negative binomial distribution with a log link. The degrees of freedom were adjusted using the Kenward-Roger method and a Tukey-Kramer multiple comparison adjustment for the p-values for the differences of LS-means were used. The GLIMMIX ilink option was used to convert means and standard errors to the data scale.

To identify a potential relationship between plasma liver enzyme or NH_3 levels and learning ability independent of the dietary treatment, a GLM model was used for each of the liver enzymes and the plasma NH_3 levels, with the ability or inability to learn as a fixed effect. A hen was considered a “learner” when she was able to successfully reach the criterion in the reversal learning task, whereas a “non-learner” was considered to be a hen that was unable to reach the learning criterion.

The results were considered statistically significant if $P < 0.05$; interactions were tested and those found not to be significant were removed from the final model. All data are presented as means ± standard errors unless otherwise indicated.

6.4 Results

6.4.1 Physiological responses: body weight, and plasma hepatic markers

Dietary treatment effects on body weight, plasma liver enzyme and NH_3 levels in weeks 5 (pre-dietary), 13 and 21 of the experiment are listed in Table 6.2. Body weight was not affected by dietary treatment (wk 13: $F_{2, 62} = 3.11$, $P = 0.0518$; wk 21: $F_{2, 59} = 2.52$, $P = 0.089$), strain (wk 13: $F_{1, 62} = 0.21$, $P = 0.652$; wk 21: $F_{1, 59} = 0.94$, $P = 0.336$) nor interaction of dietary treatment and strain during wk 13 and wk 21. Concentrations of AST, GGT, ALP and NH_3 were not affected by dietary treatment (AST: $F_{2, 43.14} = 0.28$, $P = 0.757$; GGT: $F_{2, 38.115} = 3.51$, $P = 0.05$, ALP: $F_{2, 26.7} = 1.44$, $P = 0.255$; NH_3: $F_{2, 62} = 0.16$, $P = 0.871$), strain (AST: $F_{1, 43.14} = 0.28$, $P = 0.757$; GGT: $F_{1, 40.03} = 2{,}28$, $P = 0.139$, ALP: $F_{1, 32.65} = 2.41$, $P = 0.130$; NH_3: $F_{1, 56} = 0.20$, $P = 0.658$), nor interaction of dietary treatment and strain (AST: $F_{2, 38.12} = 1.89$, $P = 0.165$; GGT: $F_{2, 45.94} = 0.68$, $P = 0.511$, ALP: $F_{2, 26.6} = 0.64$, $P = 0.534$; NH_3: $F_{2, 1} = 0.03$, $P = 0.971$) and did not change over time (AST: $F_{2, 39.07} = 0.668$, $P = 0.570$; GGT: $F_{2, 41.98} = 1.37$, $P = 0.266$, ALP: $F_{2, 26.6} = 0.64$, $P = 0.534$; NH_3: $F_{2, 1} = 0.12$, $P = 0.898$).

Table 6.2. Plasma liver enzyme activity (U/L), plasma NH_3 concentration (µg/mL), and body weight (kg) at wk 5 (start of the dietary treatment = no dietary treatment), wk 13 (eight weeks after dietary treatment), and wk 21 (16 weeks after dietary treatment).

Variables[1]	Dietary Treatments		
	Control (18% CP)	16% CP	12% CP
AST,			
wk 5	187.71±17.305*(n=21)*	172.04±9.792*(n=25)*	168.68±10.318*(n=25)*
wk 13	155.80±11.947*(n=19)*	162.91±11.273*(n=24)*	150.51±10.635*(n=25)*
wk 21	146.90±11.996*(n=19)*	155.72±10.063*(n=22)*	146.59±10.367*(n=21)*
GGT,			
wk 5	107.85±14.144*(n=21)*	118.60±14.483*(n=25)*	92.04±11.498*(n=25)*
wk 13	121.58±30.279*(n=19)*	47.32±11.175*(n=24)*	58.13±14.463*(n=24)*
wk 21	139.94±39.829*(n=19)*	110.82±24.535*(n=23)*	78.63±19.326*(n=23)*
ALP,			
wk 5	325.26±30.085*(n=19)*	402.91±32.261*(n=24)*	343.35±31.840*(n=25)*
wk 13	287.25±76.468*(n=19)*	276.16±59.740*(n=24)*	231.46±54.211*(n=25)*
wk 21	377.02±113.74*(n=19)*	240.79±50.278*(n=23)*	184.17±39.652*(n=23)*
Plasma NH_3,			
wk 5	2.15±0.057*(n=21)*	2.74±0.112*(n=25)*	2.32±0.047*(n=25)*
wk 13	2.63±0.387*(n=19)*	2.45±0.338*(n=24)*	2.84±0.340*(n=25)*
wk 21	2.51±0.378*(n=19)*	2.54±0.348*(n=23)*	2.79±0.368*(n=22)*
Body weight,			
wk 5	2.23±0.040*(n=21)*	2.18±0.048*(n=25)*	2.15±0.041*(n=26)*

wk 13	2.25±0.334*(n=20)*	2.21±0.303*(n=24)*	2.19±0.297*(n=25)*
wk 21	2.29±0.337*(n=19)*	2.25±0.306*(n=24)*	2.25±0.301*(n=24)*

[1]AST = Aspartate Aminotransferase; GGT = Gamma-glytamyl Transferase; ALP = Alkaline Phosphatase

6.4.2 Behavioural observations

6.4.2.1 Discrimination task (before the dietary treatment started)

Fifty-seven out of 73 birds (78%) were able to associate colour and dislodge a fully covered well by sliding the lid (curved-sliding) back to get access to a reward five out of six trials on two consecutive days. On average, the number of sessions required to achieve the learning criterion was 1.6±1.29 (color association task), 2.8±1.31 (when the reward was partially covered), and 4.7±2.83 (during the novel task of completely dislodging the lid to access the reward). The number of sessions needed to advance stages (reward uncovered, reward partially covered) or complete (reward fully covered) the discrimination testing revealed no difference ($P > 0.05$) between strains (Table 6.3). Interestingly, white-feathered birds needed more trials than brown-feathered hens when the reward was uncovered ($F_{1, 64} = 3.76$, $P = 0.05$) and when reward was partially covered ($F_{1, 61} = 6.80$, $P = 0.01$) (Table 6.4).

6.4.2.2 Reversal discrimination task

Fifty-seven out of 73 birds performed the reversal discrimination task. Forty-four out of 57 birds (77%) were successful in reaching the criteria of the reversal discrimination task. On average, the number of sessions required to achieve learning criterion during reversal discrimination task was 12.8±2.94. The number of sessions were not affected by dietary treatment (Control: 13.63 ± 0.917 vs. 16% CP diet: 12.89 ± 1.391 vs. 12% CP diet: 12.7 ±

0.904; $F_{2, 33}$ = 0.23, *P* = 0.793), nor genetic strain ($F_{1, 33}$ = 0.19, *P* = 0.667), nor were any interactions observed (Table 6.3). The number of trials were not affected by dietary treatment (Control: 75.56±5.519 vs. 16% CP diet: 85.49±5.242 vs. 12% CP diet: 76.90±4.454; $F_{2, 44.56}$ = 1.04, *P* = 0.362). However, white-feathered birds needed a higher number of reversal trials than brown-feathered birds ($F_{1, 44.71}$ = 6.37, *P* = 0.015) (Table 6.4). No interactions between dietary treatment and strain were observed regarding the number of trials.

As shown in Table 6.5 plasma hepatic enzymes and NH_3 concentrations were not related to achieving the learning criteria, except ALP enzyme, which was significantly elevated in non-learners compared to learners (T = 7.18; *P* < 0.009) during the baseline discrimination task.

Table 6.3. Effect of brown- and white-feathered hens on the number of sessions during behavioural testing

Strain	Discrimination testing (baseline)			Discrimination reversal testing
	Colour association	Reward partially covered	Reward fully covered	
Brown-feathered	1.18±0.313*(n=34)*	2.20±0.418*(n=34)*	5.22±0.711*(n=33)*	12.48±0.993*(n=30)*
White-feathered	2.19±0.639*(n=34)*	3.52±0.752*(n=34)*	3.53±0.639*(n=24)*	13.45±1.587*(n=14)*
Significance	0.24	0.42	0.17	0.66

n = number of birds successfully reaching the learning criterion of the task

Table 6.4. Effect of brown- and white-feathered hens on the number of trials during behavioural testing

Strain	Discrimination testing (baseline)			Discrimination reversal testing
	Colour association	Reward partially covered	Reward fully covered	
Brown-feathered	7.35±0.761*(n=34)*	13.75±1.015*(n=34)*	25.77±2.636*(n=33)*	70.57±3.636*(n=30)*
White-feathered	10.07±	18.57±1.413*(n=34)*	31.15±3.552*(n=24)*	88.88±5.776*(n=14)*
Significance	0.05	**0.01**	0.25	**0.01**

n = number of birds successfully reaching the criterion of the task

Table 6.5. Plasma enzyme (U/L) and ammonia (µg/mL) concentrations in laying hens during the discrimination and reversal discrimination task.

Variable	Learners	Non-learners	P-value
AST	176.78±8.168*(n=57)*	169.71±16.480*(n=16)*	0.7017
	162.40±11.407*(n= 44)*	***162.27±22.291*** *(n=13)*	0.9958
GGT	110.61±8.804*(n=57)*	87.57±17.766*(n=16)*	0.2492
	125.95±15.374*(n=44)*	***113.18±30.041****(n=13)*	0.7067
ALP	326.80±20.569*(n=57)*	450.07±41.139*(n=16)*	**0.0092**
	248.41±24.273*(n=44)*	***329.90±47.991****(n=13)*	0.1358
Plasma NH_3	2.43±0.061*(n=57)*	2.37±0.124*(n=16)*	0.6302
	2.65±0.054*(n=44)*	***2.65±0.106****(n=13)*	0.9919

AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; ALP = Alkaline phosphate; Learners = birds that were able to reach the learning criterion of 5 out of 6 successful trials over two consecutive days; Non-learners = birds that were unsuccessful in reaching the learning criterion. Values in ***bold and italics*** refer to the level of plasma enzymes and ammonia in learners and non-learners for the discrimination reversal learning task.

6.5 Discussion

The study investigated the effect of LPER diet consumption on plasma markers of hepatic damage and performance in a reversal discrimination task in adult laying hens. No significant difference was found in the body weights in the hens fed LPER diets. No change in body weight is consistent with the study by Rozenboim et al. (2016) in 80 weeks old hens fed LPER diets; however, the same study reported reduced body weight in younger hens fed LPER diets. Some studies suggest low dietary protein results in

reduced body weight (laying hens: Bunchasak et al., 2005; Yakout, 2010, broilers: Bregendahl et al., 2002; Aletor et al., 2000) while others suggest no impact on body weight (laying hens: Meluzzi et al., 2001; Rozenboim et al., 2016). The impact of dietary protein on adult hens may not be the same as on young hens since younger hens have additional protein requirements to support body growth and egg production and are likely to be more sensitive to dietary changes (Parenteau, 2019). Therefore, the lower impact of dietary change in adult hens might explain why body weight was not affected as a result of change in dietary protein levels. Additionally, lowering dietary protein could have resulted in lower egg production and/or lower egg weight to compensate for the protein requirement of adult hens (Shim et al., 2013). However, egg production and egg weight was not measured in this study.

No differences were found in AST, ALP, GGT activity levels and plasma NH_3 after 16 weeks of feeding 16% CP diet and 12% CP diet compared to Control diet. These findings are unexpected. Previous studies have found that low protein diets and excessive energy intake results in the increased level of plasma enzymes indicating hepatic damage leading to an increased level of blood NH_3 (Butler, 1976; Rozenboim et al., 2016). The use of AST, ALP, and GGT activity to determine liver damage has been indicated in several studies (Diaz et al., 1999; Jaensch et al., 2000; Yousefi et al., 2005; Zhang et al., 2008; Shini, 2014; Rozenboim et al., 2016). However, Rozenboim et al. (2016) suggested that plasma enzyme activities of ALP, GGT, and AST could be a poor diagnostic tool of liver damage in commercial strains of hens as their study did not find consistently higher plasma levels of liver enzymes throughout the experiment in hens fed low-protein high-

energy diets. On the other hand, for strains UCD-003, Single Comb White Leghorns predisposed to FLHS, Diaz et al. (1999) found consistently higher activities of enzymes indicating liver damage. Nevertheless, von Waldburg-Zeil et al. (2018) could not replicate these results in the UCD-003 strain. In the current study, differences might not have been observed as plasma enzyme activities fluctuated across weeks even in the same group of hens fed the same diet. Furthermore, the observation of hepatic failure requires the damage of the majority of hepatic parenchyma (Hochleithner et al., 2006), which may require a duration beyond that of this study.

Considerably higher levels of plasma GGT were observed in the present study compared to the studies conducted by Shini and Bryden (2009) and Shini (2014). Birds used in Shini (2014) were younger than the birds used in this study. Harr (2006) mentioned that older birds can have slightly higher GGT values than young ones. This could explain why hens in this study had higher GGT levels than those previously reported in the literature. Further, a covert form of hepatic damage cannot be ruled out in the age class used in this study, which could not be determined by the results obtained from plasma enzyme levels indicating hepatic damage. It may be that no single test is enough to assess overall liver function *in vivo,* and the best interpretations are achieved using the integrative approach of combining physical exams, imaging, blood tests, biopsies and post-mortem analyses (Hochleithner et al., 2006; Center, 2007; Shini, 2014).

No difference was observed across the three different diets in discrimination reversal learning in hens. Similar to the study reported here, Bona et al. (2018) found no impairment in the learning ability of female broilers fed LPER diets. However, studies

conducted with murine models (Aguilar et al., 2000; Wesierska et al., 2006; Rodrigo et al., 2010) have consistently demonstrated impairment in cognitive flexibility due to diet-induced hyperammonaemia and subsequent liver pathogenesis. In chickens, much of the brain development and maturation occurs at or before the age of 10 weeks (Atkinson et al., 2008). The hens used in the current study had a fully developed brain, which could have been one of the reasons why the interventions in diet did not affect the brain and subsequent behaviours displayed by them. Additionally, before the experiment, the hens were kept on a commercially recommended diet until 72 weeks of age, by which most of the physical, neuronal and behavioural development had taken place in hens. To further explore this, testing is suggested in younger, as well as older hens. Additionally, different personality traits of hens should also be considered as individual intellectual ability and motivation to learn task can affect learning ability in hens (Herborn et al., 2014).

In this study, hens having higher ALP enzyme activity were less successful in learning baseline behavioural tasks compared to birds having lower ALP enzyme activity. In birds, increased ALP activity has been associated with the increased osteoblastic activity (Harr, 2006). Today's laying hens have poor skeletal health and a high incidence of keel bone damage (up to 90% of hens) likely associated with pain leading to compromised welfare (Harlander-Matauchek et al., 2015). In chickens, osteoblastic activity during bone healing is associated with elevated ALP concentrations and used as a marker for evaluating skeletal healths (Lumeij and Westerhof, 1987; Jiang et al., 2013). Osteoblastic activity due to bone damage (e.g. keel bone damage) is associated with pain (Gebhardt-Henrich et al., 2017). Further, pain is associated with reduced cognitive processing in mammals

(Nadar et al., 2016). Similar considerations could have happened in laying hens of this study. Additionally, elevated plasma ALP concentrations partly mirror neuronal loss in the brain, which is correlated with cognitive impairment in humans (Kellet et al., 2011). This might partially explain why birds with higher plasma ALP activity were inferior in learning; however, this needs further hypothesis-driven testing.

Interestingly, the feather colour (white/brown) of birds affected hens' learning ability during both initial motor and visual discrimination task and the reversal task. Brown-feathered hens achieved learning criteria earlier than white-feathered hens in general. Strain differences in learning are also common in other species, most notably mice (Colacicco et al., 2002; Novak et al., 2016). The reasons behind white-feathered hens performing inferior to brown-feather hens could be their fearfulness of the test setting as indicated by various studies (Jones, 1996; Albentosa et al., 2003; Fraisse and Cockrem, 2006; de Haas et al., 2013; de Haas et al., 2017). Additionally, brown-feathered hens demonstrated more exploratory behaviours (personal observation) than white-feathered hens, which might have affected their learning ability.

The limitations of this study should be considered to better understand the results. The average feed intake per hen per pen, differences in macronutrient composition (protein and energy), as well as the discrepancies in the nutrient requirements of individuals could affect the power of this study to achieve significant differences. The hens in this study were reused from other behavioural trails and were the only ones available for the study at the time of experimentation. These hens had a previously established social structure, therefore mixing would induce stress, which would affect their learning behaviour (Birkl et

al., 2019). However, the results presented in this study should be understood from a behavioural standpoint, as focus was laid on acquiring behavioural data from hens induced with FLHS. Additionally, plasma hepatic enzymes other than ALP, AST, and GGT (e.g. alanine aminotransferase - ALT) were not measured. Had ALT been measured, AST:ALT ratio could be calculated that has been shown to indicate liver damage in humans (Chitturi and Farrel, 2013). Moreover, using plasma hepatic markers to refine hepatic damage diagnosis meant that liver damage was not confirmed directly through necropsy. Rozenboim et al. (2016) observed increased hemorrhagic score in the post-mortem examination of young hens fed LPER diets and also reported hemorrhagic score and liver colour score to be higher in older hens compared to younger one, suggesting aging to be a major factor affecting hen's liver. This indicates that age of hens could have influenced these results. Future studies are suggested to incorporate necropsy findings along with other indicators such as plasma hepatic markers and physical exams.

In conclusion, a lower protein and energy-rich diet does not impair learning ability in adult laying hens. Also, such diets do not impact liver health and body weight in adult laying hens as indicated by the data obtained in current experimental situations. These findings could have implications to the environment as low protein in the diet is associated with low nitrogen load to the environment. There might be the possibility of using such diets without necessarily impacting birds' weight and liver health. This study generated interesting questions regarding ALP enzymes, which was associated with poor learning in hens warranting further study.

6.6 Acknowledgements

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7 General Discussion

This chapter is divided into five sections that provide a full synopsis of the thesis. The first section (7.1) concerns the background of why and how this project began. The second section (7.2) lists and discusses the thesis' key findings. The third section (7.3) entails the thesis' strengths and the fourth section (7.4) presents limitations and afterthoughts that will form the basis for future directions. Finally, this thesis will be concluded in the fifth section (7.5) with a take-home message for the reader.

7.1 Background

Before this study began, there was no abundant source of published information on the impact of air/excreta gas mixtures on the behaviour of laying hens. The available studies were primarily focused on only one excreta gas, i.e. NH_3 (Gholap, 2012; Kristensen et al., 2000; Drake et al., 2010). Studies had emphasized the adverse effects of excreta and wet litter on damage to integument (Wang et al., 1998), the ability of the bird to avoid excreta gas (mainly NH_3) (Kristensen et al., 2000; Wathes et al., 2002), and how NH_3 can be reduced in poultry farms (Sutton et al., 2001; Roberts et al., 2007).

The main conclusions from these studies were: high concentration of NH_3 negatively impacts laying hens' health and welfare (Amer et al., 2004; Lay et al., 2011); laying hens avoid NH_3 concentration above 20-25 ppm (Kristensen et al., 2000; Wathes et al., 2002); and NH_3 production in poultry farms can be reduced by using litter amendments such as poultry litter treatment (PLT®) and by feeding lower dietary protein to laying hens (Nahm, 2007; Choi and Moore, 2008; Veens et al., 2009). All these studies were significant in

contributing to knowledge about laying hens' behaviour and nutrition. Nonetheless, several questions emerged after carefully reviewing those studies. While the studies were conducted with a focus on NH_3, the researchers discounted the excreta itself. There was also a gap in how excreta exposure affects laying hens' behaviour. Similarly, how NH_3-reducing techniques, such as PLT® and feeding low dietary protein, affect laying hens' behaviour and physiology remained unknown.

Meanwhile, there was a pressing demand from the public sector - advocacy groups such as Humane Society International, retail chains such as McDonald's and Tim Hortons, and agencies such as Egg Farmers of Canada - to move from conventional cage systems towards alternative housing systems. This signified the impending emergence of other health and welfare issues in litter-based housing systems, such as the possibility of increased excreta gas production (Wheeler et al., 2003) and exposure to excreta and excreta-soiled litter substrate (EFSA, 2005).

Therefore, it was essential to answer questions concerning laying hen excreta and its impact on hens' behaviour. Two main aspects were considered: hens' behavioural reaction to excreta-soiled environments (chapters 3, 4, and 5) and the effect of excreta gas-control strategies (PLT® and low-protein diets) on hens' behaviour and physiology (chapters 5 and 6).

At first, a gaseous environment was created directly from excreta to study its impact on hens' behaviour (Chapter 3). Then, the impact of excreta exposure on hens was investigated using excreta-soiled scratch pads as a tool in enriched cages (Chapter 4).

Further investigation was carried out to find whether hens would be attracted to or avoid excreta-soiled litter substrate in floor pens that resembled litter-based housing systems (Chapter 5). Finally, the impact of nitrogen-reducing diets on laying hens was investigated to understand the impact of such diets on their behaviour and physiology (Chapter 6).

7.2 Key findings

7.2.1 Chapter 3 (Laying hens behave differently in artificially and naturally sourced ammoniated environments)

The key findings of Chapter 3 are that laying hens prefer foraging in the fresh air; they show aversion towards rising NH_3 concentrations, and they behave differently in NH_3 environments created artificially and from laying hens' excreta (natural source). The excreta-sourced ammoniated environment was a more familiar stimulus to laying hens compared to the artificial source, which could have caused a lesser degree of aversion in hens. The display of aversive response towards high NH_3 concentration was consistent with the findings of previous studies (Wathes et al., 2002; McKeegan et al., 2005). These findings have implications regarding the recommendation of NH_3 levels in poultry houses, as previous behavioural and physiological studies on the negative impacts of NH_3 on poultry were based on NH_3 created from artificial sources.

Our intention in this study was to explore the possibility of excreta having other factors than NH_3 that affect laying hens' behaviour. Results indicated that excreta, by itself, could elicit a different response in hens compared to NH_3 alone. However, the extent to which excreta itself affects laying hens' behaviour was yet to be understood. Therefore, another

study (Chapter 4) was designed to investigate the holistic impact of excreta on laying hens' behaviour.

7.2.2 Chapter 4 (How does the presence of excreta affect the behaviour of laying hens on scratch pads?)

Herein, the behaviour of laying hens on excreta-soiled scratch pads was studied. Interestingly, laying hens showed relative preference to excreta-soiled scratch pads compared to unsoiled ones. This finding is notable from a behavioural point of view, as it suggests laying hens use excreta when no other foraging material is available. Laying hens foraging on their own excreta could also be attributable to the nutritional benefits of foraging/feeding on excreta (McGowan, 1995; Negro et al., 2002). However, there could be short- and long-term impacts of foraging/feeding on excreta, which are worth investigating. Additionally, the implications that excreta exposure can have on hygiene (e.g. egg contamination) and health (e.g. footpad dermatitis) are other facets that are yet to be understood (Rodenburg et al., 2005; Van Staaveren et al., 2018).

The findings from Chapter 4 have relevance to the egg industry, which is in a transition phase from conventional cage systems to alternative housing systems (EFC, 2016). Additionally, this also has significance for consumer perspectives regarding the availability of substrates to promote the expression of natural behaviours such as foraging.

7.2.3 Chapter 5 (Do laying hens differentiate among excreta-soiled and unsoiled litter substrates?)

As hens are exposed frequently to excreta-soiled litter substrates in litter-based housing systems, Chapter 5 was designed to understand whether hens have an affinity for or avoid excreta-soiled litter substrates. Herein, hens' relative preference for excreta-soiled litter substrates, PLT® treated excreta-soiled litter substrates, and unsoiled litter substrates was investigated. Hens were expected to prefer unsoiled litter substrates and PLT® treated excreta-soiled litter substrates to excreta-soiled litter substrates. However, the hens did not show a relative preference toward either of these litter substrates. In the previous chapter (Chapter 4), hens had a relative preference to the excreta-soiled environment, whereas, in this study, the hens were indifferent towards excreta-soiled or unsoiled substrates. This suggests that substrate availability itself is more important to hens than the quality of the substrate, which aligns with the findings of previous studies that hens are motivated to access litter substrates (Dawkins, 1983; Matthews et al., 1995). However, these two experiments (chapters 4 and 5) were conducted in two different housing systems (the former in an enriched cage and the latter in a litter-based housing system), which might have affected hens' behavioural response. Findings from Chapter 5 further strengthen the idea of transitioning from conventional cage systems to alternative litter-based housing systems. Additionally, the ineffectiveness of PLT® in reducing litter pH in this study suggests that the optimum effect of PLT® can be achieved only when litter moisture is sufficient (Shah et al., 2012).

7.2.4 Chapter 6 (Effect of low-protein energy-rich diet on body weight, plasma hepatic markers, hepatic damage and learning ability of adult laying hens)

In chapters 3, 4, and 5, the impact of excreta/excreta gas exposure on laying hens' behaviour was investigated. As stated previously, there is not much information on how excreta gas (mainly NH_3) reducing strategies such as dietary interventions affect laying hens' behaviour and physiology. Therefore, in Chapter 6, the effect of low-protein and energy-rich diets on hepatic markers indicating liver damage and learning abilities in hens was investigated. There was no impact of low-protein energy-rich diet on plasma hepatic markers indicating liver damage and learning abilities in hens. This implies that there might be the possibility of using such diets without negatively impacting hens, which could be beneficial both economically and environmentally (Morse, 1995; Robinson and Singh, 2001; Bregendahl et al., 2002). Interestingly, learning was influenced by the feather colours of hens, as brown-feathered hens learned faster than white-feathered hens. These differences in learning might have implications while housing hens in complex housing systems that demand behavioural flexibility and quick learning ability. Additionally, hens with higher levels of plasma alkaline phosphatase (ALP) were less successful learners than those with lower ALP. This is a significant finding obtained from this study, as higher ALP has been associated with cognitive impairment in humans (Kellet et al., 2011). Further studies are recommended to understand more about the relationship between elevated ALP activity and learning ability in hens.

7.3 Strength of this thesis

This thesis has several key strengths. It presents a number of unique methods for behavioural testing in hens. In Chapter 3, a custom-built polycarbonate chamber (Figure 3.1) was used with an opportunity to manipulate gaseous exposure to hens. To the knowledge of the author, this is the first study to test hens' behavioural reactions to NH_3 generated from laying hens' excreta. In Chapter 5, hens' motivation to access soiled or unsoiled litter substrates was assessed using a push door apparatus. The push door (Figure 5.2) was built using polycarbonate plastic, allowing a clear vision of the litter substrate treatments on the other side. Hens had no difficulty in using these doors. Therefore, such doors could be used in future behavioural studies of similar nature. In Chapter 6, a unique behavioural testing apparatus (Section 6.3.4.1) was used to test the laying hens' learning ability. This testing apparatus had two grooves, each covered by a circular disc with the ability to slide the disc to conceal a food reward kept in the grooves. The design required a response from hens not only in the form of visual discrimination between two colours (green and red) but also a deviation from their natural foraging strategies. Hens had to use different strategies to move the circular disc, such as using their beak to pull the disc, tipping the disc, and scratching the disc. Such a design allowed hens to successfully perform a novel motor task, indicating their cognitive flexibility.

7.4 Afterthoughts/future directions

Although the results from Chapter 3 could be valuable in reappraising the standards regarding NH_3 concentrations in laying hen houses, there are several things I would be interested in reconsidering if this study was to be repeated. Gases other than NH_3 can

impact hens' behaviour (CO_2: Blackshaw et al., 1988; McKeegan et al., 2005 H_2S: McKeegan et al., 2005). Therefore, I would like to explore opportunities to fully characterize gaseous environments inside the experimental chamber. I would also measure physiological responses (e.g., heterophil: lymphocyte ratio, fecal corticosteroid) to understand the stress response in hens exposed to aversive conditions such as excreta gases. Further, to understand whether hens were reacting aversively to NH_3 or just reacting to a new odour, I would like to add another control using a different odour. Regarding experimental design, I would like to place hens in an already ammoniated environment and observe their attempts to avoid such an environment. The effect of competing motivations (food rewards and the aversive gaseous environment) could be avoided using that approach.

Chapter 4 generated important questions on the consequences of excreta exposure in laying hens. However, from the results, it can be questioned whether hens were responding to excreta or feed or a combination of both. If I had the opportunity to repeat the experiment, I would be interested in adding additional treatments (e.g. excreta alone without a feed from the auger, scratch pads with the neutral substrate, and scratch pads of different colour).

Chapter 5 helped in understanding hens' preference towards excreta-soiled litter substrates. The results showed that hens were indifferent towards excreta-soiled or unsoiled litter substrates. The National Farm Animal Care Council, which is responsible for providing guidelines for housing laying hens in Canada has mentioned that litter can be "the mixture of excreta, feathers, feed, dust and other materials on the floor" with or

without bedding materials (NFACC, 2017). That means farmers are allowed to use a mixture of excreta, feathers, feed, and dust as a litter material when litter is required. What this means to hens from the behavioural point when only excreta is used as a litter substrate is not clear. If this study is to be repeated, I would have liked to add the mixture of excreta, feathers, feed, dust, and other materials (except bedding) on the floor as an additional treatment, which would have helped in revisiting the definition of litter by NFACC (2017). The results of chapter 3 and chapter 5 suggest that hens can use excreta or excreta-soiled substrates as a foraging material. Whether it was due to their motivation to access these resources or due to the consequence of not having other desired resources is yet to be understood and should be considered in future studies. Additionally, I would have tested hens in isolation to compare the results obtained from group testing. Use of greater range of resources and testing both in isolation and in group would have increased the validity of this study and allowed these results to be extrapolated to different situations.

Chapter 6 contributed to adding further information on plasma hepatic markers indicating hepatic damage. Generally, there is a lack of information about reference values on avian plasma hepatic markers indicating liver damage (Yap and Aw, 2010). If this study is to be repeated, I would like to measure a muscle enzyme, creatine phosphokinase (CPK). Hochleithner (2006) suggested that AST levels should be analyzed together with CPK as AST is also released from damaged muscle tissues and the odds of liver damage is higher if only AST is elevated out of these two enzymes.

7.5 Overall conclusions

This study serves as a valuable step in understanding hens' preference and avoidance towards excreta-polluted conditions. As the laying hen industry is marching to the litter-based housing systems, the results presented here give invaluable guidance to farmers, poultry researchers, and the relevant authorities. Based on the results presented here, it can be concluded that hens prefer to forage in the fresh air, avoid ammoniated environments, and use their own excreta as a foraging material when other foraging options are not available. These results solidify the previous findings that litter substrates are essential resources for laying hens. Further, the use of PLT® as a litter amendment may not be effective when litter moisture is insufficient. Finally, the results presented indicate that nitrogen-reducing diets do not impair learning in laying hens, implying that such diets could be more sustainable economically and environmentally.

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