Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
Thesis 2023:82

Choosy chicks: Chicken life quality is a matter of choices

Selektive kyllinger: Kyllingens livskvalitet er et spørsmål om valg

Regine Victoria Holt

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This journey did not go quite as planned, but thanks to all of these amazing people around me, I still managed to complete it.

"Life is a matter of choices, and every choice you make makes you."
– John C. Maxwell

Table of Contents

1 Definitions

Definitions

Term	Meaning
ad libitum	free; as much or as often as necessary or desired
animal(s)	non-human animal(s)

2 List of papers

The thesis is based on the following papers:

Paper I:	Holt, R.V., Vas, J., Vasdal, G. & Newberry, R.C. (2023). A buffet of litters – Broiler chickens behave differently according to litter type and freshness. *Applied Animal Behaviour Science*, 260: 105856. https://doi.org/10.1016/j.applanim.2023.105856.
Paper II:	Holt, R.V., Skånberg, L., Keeling, L.J., Estevez, I. & Newberry, R.C. (2023). Resource choice during juvenile development contributes to long-term welfare in laying hens. [Manuscript].
Paper III:	Holt, R.V., Skånberg, L., Keeling, L.J., Estevez, I., Lepej, P. Andersen, I.L., Vas, J. & Newberry, R.C. (2023). Comb size, shape complexity, and laterality of laying hens reared in environments varying in resource choice. [Manuscript].

3 Abstract

In the recent years, the use of environmental enrichments has increased, with beneficial effects on animal welfare. However, many of the documented effects of environmental enrichment have been improvements on the negative end of the welfare scale, identifying environmental features necessary to reduce harm rather than necessarily improving quality of life on the positive side. One potential way of enhancing positive aspects of animal welfare would be to offer additional choice. Broilers and laying hens (*Gallus gallus domesticus*) are among the world's most common production animals, and are often kept indoors in intensive production facilities. Offering them choice by providing resources in a variety of different forms rather than a single form could accommodate differences in individual preferences, add opportunities for cognitive decision-making, enhance skills in exploiting resources, and promote behavioural flexibility and emotional resilience when challenged by novelty, thereby contributing to a greater perception of freedom, agency and control over the environment. Ultimately, these positive experiences may be associated with a reduced accumulation of allostatic load and enhanced long-term fitness. The aim of this thesis was therefore to examine effects of providing more complex environments offering opportunities for choosing among a variety of resources on the behaviour and welfare of poultry. This topic was investigated by providing broilers with multiple litter options and laying hen pullets with multiple litter and perch types. Our results show that offering choice during rearing had positive effects on poultry welfare, some of which lasted into adulthood. Broiler chickens displayed different behavioural responses in different litter types, and a strong attraction to fresh litter, showing the value of providing them with a variety of substrates and refreshing each type regularly. Furthermore, providing laying hen pullets with multiple as opposed to single resource forms during different phases of the rearing period resulted in increased expression of positively valenced behaviours including play in early life, and dustbathing when older. It also reduced expression of negatively valenced behaviours including feather pecking during rearing and aggression in adulthood. These behavioural results were associated with better plumage condition and higher body weight in adulthood, indicative of reduced allostatic load and improved fitness. However, this did not alter the physical characteristics of the condition-dependent comb, as measured by comb size, shape complexity and laterality at peak of lay.

Collectively, these findings are consistent with the hypothesis that offering multiple resource choices represents true environmental enrichment, moving the dial from moderate to high welfare on the positive end of the welfare scale rather than from poor to moderate welfare on the negative end. In conclusion, providing a complex environment offering multiple resource choices has both short- and long-term positive effects on poultry behaviour and welfare. The findings in this thesis provide a biological foundation for motivating farmers to provide environmental enrichments that enhance their animals' positive experiences.

4 Norsk sammendrag

I senere år har bruken av miljøberikelse økt og ført til gunstige effekter for dyrevelferd. Likevel har mange av de dokumenterte effektene vært forbedringer på den negative siden av velferdsskalaen hvor miljømessige faktorer har blitt identifisert i stedet for å forbedre livskvaliteten på den positive siden av velferdsskalaen. En potensiell metode å øke positiv dyrevelferd kan være å tilby et mer variert miljø med valgmuligheter. Slaktekylling og verpehøns (*Gallus gallus domesticus*) er blant verdens vanligste produksjonsdyr, og holdes somoftest innendørs i intensive produksjonsanlegg. Å tilby valgmuligheter ved å gi fjørfe flere ulike ressurser, istedenfor bare en enkelt type, kan: imøtekomme individuelle preferanser, legge til rette for kognitive beslutninger, forbedre ferdigheter i bruken av ressurser, fremme fleksible og robuste individer i møter med nye utfordringer. Dette kan dermed bidra til en større opplevelse av frihet, innflytelse og kontroll over miljøet de lever i. Til syvende og sist kan disse positive opplevelsene være assosiert med en redusert stress belastning og forbedret langsiktig velferdsmessig tilstand. Målet med denne avhandlingen var derfor å undersøke effekten av å tilby mer komplekse miljøer med muligheter for å velge mellom ulike ressurstyper på atferden og velferden hos fjørfe. Dette ble undersøkt ved å gi slaktekyllinger valg mellom ulike typer strø, og verpehøner valg mellom ulike typer strø og vagler. Våre resultater viste at muligheter for valg under oppveksten hadde en positiv effekt på dyrevelferd, og noen av disse varte også inn i voksen alder. Slaktekyllinger viste ulike atferdsresponser avhengig av strøtype og en sterkere tiltrekning til ferskt strømateriale, noe som viser verdien av å gi slaktekyllinger ulike substrater og regelmessig fornye hver av dem. Videre førte det å tilby verpehøns miljøer med flere ressursvalg i ulike faser av utviklingsperioden, til økt forekomst av positivt atferder, inkludert lek i tidlig utvikling og mer strøbading når de ble eldre. Det førte også til færre negative atferder, inkludert redusert fjørplukking i utviklingsperioden og aggresjon i voksen alder. Disse atferdsresultatene var også assosiert med bedre fjørdrakt og høyere kroppsvekt i voksen alder, som indikerer redusert stress belastning og forbedret velferd. Likevel ble det ikke funnet noen effekter på de morfologiske egenskapene på kammen, slik som den ble målt via kamstørrelse, formkompleksitet og lateralitet når egglegging var på topp.

Samlet er disse funnene i tråd med hypotesen om at tilbud om ulike ressursvalg representerer en god miljøberikelse og flytter velferdsskalaen fra moderat til høy velferd, istedenfor fra dårlig til moderat i den negative delen av skalaen. Konklusjonen er at å tilby et komplekst miljø med flere valgmuligheter har både kortsiktige og langsiktige positive effekter på atferden og velferden hos fjørfe. Funnene i denne avhandlingen gir en biologisk basis for å motivere bønder til å tilby miljøberikelser som forbedrer dyrs positive opplevelser.

5 Synopsis

5.1 Introduction

5.1.1 Animal welfare

Historically, the field of animal welfare has focused on reducing negative indicators of welfare, such as mortality, stress, fear, and pain. An example is the widely recognised concept of the "Five Freedoms" (Farm Animal Welfare Council, 1979), which has been used as a framework for establishing minimum standards for the protection of animal welfare. These minimum standards have been implemented into the laws and regulations of many countries (e.g. Council Directive 98/58/EC Concerning the Protection of Animals Kept for Farming Purposes, 1998; Lov Om Dyrevelferd, 2009) in response to public concerns about the impact of intensive housing conditions on farm animal suffering (Harrison, 1964; Lindgren & Forslund, 1990). However, the absence of suffering and distress is no longer considered enough to qualify as good animal welfare (Boissy et al., 2007; FAWC, 2009; Mellor et al., 2020). As a result, we should further develop our scientific understanding of animal welfare and implement enhancements rather than only precautionary actions.

Attention to the importance of facilitating positive experiences, in addition to avoiding distress, was raised in two influential publications in the early 2000's (Boissy et al., 2007; FAWC, 2009). The new approach resulted in the development of animal welfare concepts focussing on enhancements rather than precautionary measures, such as the modified "Five Domains" model (Mellor & Beausoleil, 2015) and "Opportunities to thrive" (Janssen et al., 2014). While the Five Domains model covers the same five elements as the Five Freedoms, it delves deeper into the positive mental state-aspects of animal welfare (Mellor et al., 2020, Fig.1; Mellor & Beausoleil, 2015). Furthermore, the Five Domains model clearly states that minimising negative welfare indicators does not necessarily result in good welfare and that environments need to stimulate rewarding behaviours to achieve good welfare (Mellor & Beausoleil, 2015). “Opportunities to thrive” also builds upon the Five Freedoms, but the concept highlights the importance of providing appropriate environments that not only allow for but also stimulate species-specific behaviours and optimal health (Janssen et al., 2014).

Furthermore, “Opportunities to thrive” states that animals should have environments that allow them to make choices and exercise control to avoid distress and display meaningful behaviour.

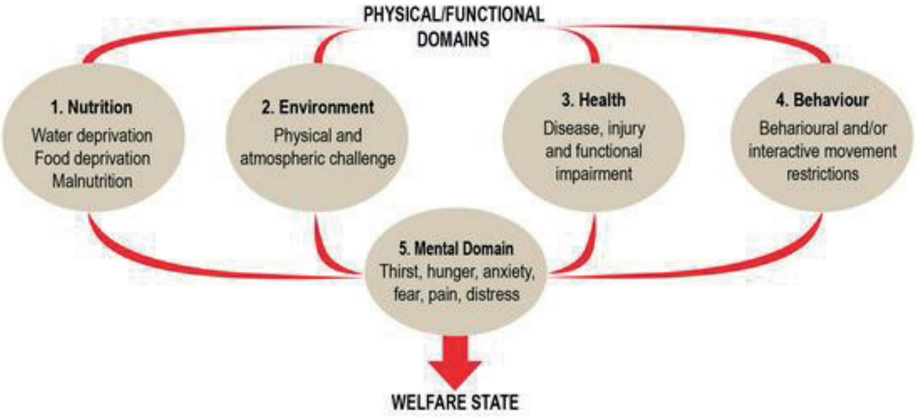

Figure 1. The Five Domains model (from Mellor et al., 2020).

In newer animal welfare concepts, provision of learning opportunities has also been highlighted (e.g. Mellor, 2017). Animal welfare should result from a balance between appropriately levelled challenges and positive experiences (Hintze & Yee, 2023; Meehan & Mench, 2007). Challenges, at an appropriate level, provide stimulation (Burn, 2017) and allow individuals to further develop their mental capacity, which may have long-term benefits. Here, the problem lies in deciding what is an “appropriate level” for challenges, especially when the mental capacity and willingness to complete a challenge may vary greatly between individuals (Hintze & Yee, 2023).

Although there is no consensus on how animal welfare should be defined (Fraser, 2009), most agree that it should be measured at the level of the individual (Richter & Hintze, 2019). However, how an individual animal perceives its environment and quality of life cannot be directly measured (Mendl et al., 2010). Recent welfare indicators aim to cover the whole spectrum of animal welfare and are often based on animal affective states, expanding upon an earlier focus on health and production measures (Balcombe, 2009).

Affective states encompass both short-term “emotions”, usually triggered by external stimuli, and the cumulative average of these emotions over time, which are called “moods”. Furthermore, affective stats include unpleasant sensations such as pain (Mendl & Paul, 2020), which are often connected to negative emotions or moods. Affective states vary dynamically according to the level of arousal and the degree of valence experienced (Mendl et al., 2010), together often referred to as “core affect” (Russell, 2003), and need not be consciously experienced (Mendl & Paul, 2020). Neurobehavioural systems connected to reward acquisition (Reward acquisition system, Fig. 2. Q1 & Q3) and punishment avoidance (Punishment avoidance system, Fig. 2. Q2 & Q4), are thought to act as proximate mechanisms that guide the individual when making decisions, thereby enhancing fitness (Burgdorf & Panksepp, 2006). These systems provide building-blocks for more complex emotional systems (Mendl & Paul, 2020).

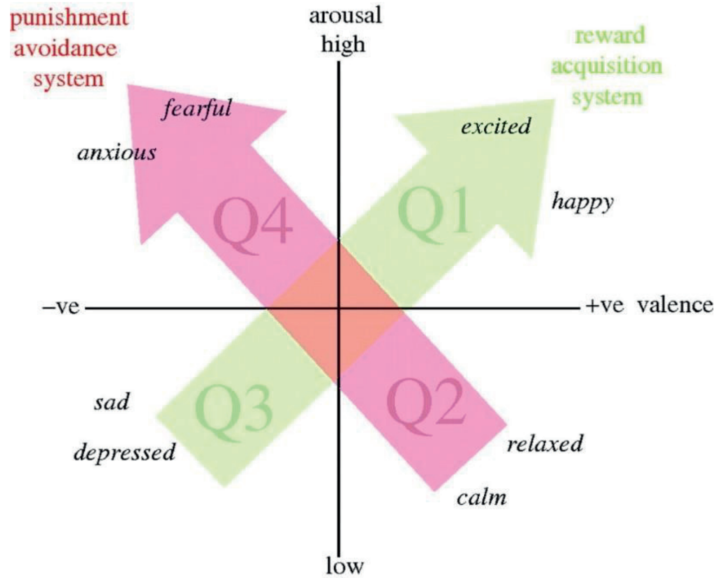

Figure 2. Dimensional core affect model from Mendl et al. (2010). The axes display the two dimensions of "Core affect": valence (negative/positive) on the x axis and level of arousal on the y axis. The arrows indicate the neuro-behavioural systems associated with reward acquisition (green) and punishment avoidance (red). Italic words indicate possible affective states and their location. Positive affective states are located in quadrants Q1 and Q2, while negative affective states are located in Q3 and Q4.

We must use welfare indicators to assess the internal mental processing of animals and make inferences regarding the positive or negative valence associated with these indicators. Generally, indicators used to assess fitness and adaptation, including self-maintenance behaviours, are considered to be positively valenced (rewarding or pleasurable) and are likely to be repeated (Fraser & Duncan, 1998; Veissier & Boissy, 2007). Likewise, indicators used to assess reduced fitness and threats to survival, including lesions, are considered to be negatively valenced (punishing or aversive) and are likely to be avoided in the future (Mendl et al., 2010). Morphological measurements, such as body weight and exterior scores (e.g. plumage or skin damage), can provide an insight into the animal's state of wellbeing. However, these measures usually require handling of the animals, which can be perceived as stressful. Behavioural observations present a non-invasive method of data collection for animal welfare assessment. Even though each individual is different and thus can have different requirements for it to thrive (Richter & Hintze, 2019), commercial farm environments are usually designed to be efficient and satisfy the basic needs of the group, not taking into account individual differences. However, adding choice to an environment might accommodate some of these differences.

5.1.2 Environmental enrichment

Captive environments are often simple, monotonous and predictable compared to the wild. Insufficient exposure to challenges and diverse stimuli in these highly efficient production conditions (Špinka & Wemelsfelder, 2011) may lead to boredom (Burn, 2017; Meagher, 2019; Wemelsfelder, 2008) and other negative impacts on animal welfare. To counter the blandness of captive environments and contribute to positive animal welfare, environmental enrichments (hereafter called "enrichments") can be provided (Meagher & Mason, 2012). These are defined as changes or additions to the environment that improve the biological functioning of the animals (Newberry, 1995). Adding enrichments increases both the diversity and the complexity of the environment, making it more stimulating by allowing for more opportunities to engage in positive species-specific behaviours (Tahamtani et al., 2020; Vas et al., 2020). Giving animals more control over their environment can also increase agency, making a profound contribution to positive welfare (Špinka, 2019).

Increasing the degree of environmental complexity and diversity usually results in better animal welfare when compared to simpler, more barren environments. With rewarding resources spread around the environment, movement is likely to increase in general (Bach et al., 2019). Ocepek et al. (2020) reported that pigs displayed higher frequencies of positive welfare indicators (more play behaviour and curly tails), in addition to lower frequencies of negative welfare indicators (less aggression and manipulation of the ears and tails of pen mates), when provided twice daily with a variety of various fresh rooting materials compared to the standard bedding material, wood shavings. However, for enrichments to have more than a fleeting positive impact on animal welfare, some prior knowledge of the target species is required as it is essential that the enrichments provided are biologically relevant to the species. Consequently, adding a random “toy” and leaving it long-term may not lead to anything more than a momentary animal welfare benefit until the novelty wears off (van De Weerd & Ison, 2019; Newberry, 1995). How the resource is presented (e.g. with or without previous experience, timing of first access, continuous or limited access, frequency of access) and maintained is also important. Abou-Ismail & Mendl (2016) found that rats provided with continuous access to enrichments displayed more positive welfare indicators than rats that had weekly rotating access to the same enrichment types. Furthermore, when enrichments get dirty, diluted or destroyed, their attractiveness drops (van De Weerd et al., 2003). Hence, while provision of environmental enrichments improves the condition of captive animals, it requires knowledge of how to best maintain the enriching value of the resources provided. Nonetheless, the potential beneficial effects of environmental enrichments are often marginally supported by the observed welfare indicators (e.g. Bach et al., 2019; Bailie & O’Connell, 2014).

Individuals differ between each other in their ways of perceiving and reacting to similar stimuli and these personality differences are usually stable over time (Biro & Stamps, 2008). Individuals within a population also vary in their responses to environmental opportunities over time in relation to differences in brain plasticity (Freund et al., 2013), and in the predictability of their responses from day to day (Goold & Newberry, 2017). As a result, at any one time, individuals varying in personality, plasticity and predictability are likely to have different preferences that facilitate thriving within their environment. In support of this hypothesis, Rufener et al. (2018) found large, consistent variation between individual laying hens in their movement between tiers of an aviary. It is therefore unlikely that in groups consisting of hundreds of individuals, the provision of a single enrichment type will benefit all members of the group equally (Richter & Hintze, 2019). Perhaps this is one of the reasons why many preference studies report inconsistent results (e.g. individual variation in litter preference in laying hens in Nicol et al., 2001). To enhance animal welfare for all individuals in a group, rather than just “the average”, the provision of more than one type of a particular resource (e.g. multiple types of litter materials) could be useful.

5.1.3 Choice provision

In this thesis, I define provision of multiple variants of a resource category as "choice" or "resource choice" (Fig. 3). Choice provision allows more opportunities for decision-making and expression of diverse behaviour (Abou-Ismail et al., 2010). Although the provision of choice implies an increase in environmental complexity or diversity, the term "choice" seems fitting as individuals may choose what resource type to use and how and when they use it. Furthermore, it is reasonable to expect that choice provision will have benefits attributed to increasing environmental complexity or diversity as providing choice serves as one approach to adding complexity and diversity to the environment.

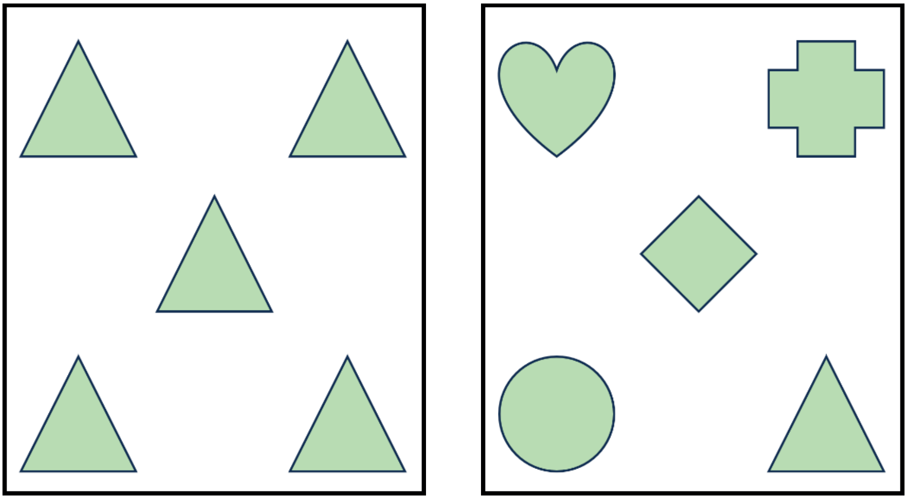

Figure 3. Example of providing a single type of an environmental enrichment (left) and providing multiple types of the same environmental enrichment (right; "resource choice"). Created by RV Holt in Microsoft 365 PowerPoint version 2306.

In addition to accounting for individual differences, providing opportunities for decision-making may be an efficient approach to improving captive environmental conditions for animals with an evolutionary history of natural selection in complex and diverse environments. Increased choice should provide more learning opportunities, thereby increasing behavioural flexibility compared to that of conspecifics living in simpler environments (Abou-Ismail et al., 2010). Increased behavioural flexibility to exploit different resources could facilitate more rapid adaptation when subsequently exposed to novelty, aiding in stress resilience. Even with abundant and dispersed resources, environments with limited diversity (provision of the same enrichment multiple times) may result in more rapidly diminishing interest and engagement compared to providing resource choice by giving multiple versions of an enrichment type (e.g., several different types of gnawing objects; Abou-Ismail et al., 2010). As different resources are used for different activities, with their current value varying depending on changing motivations (de Jong et al., 2007), a lack of choice could limit the satisfaction of fulfilling behavioural motivations and negatively affect long-term emotional and physical development. Furthermore, the lack of choice in an impoverished animal environment may contribute to redirection of behaviour to inappropriate targets (e.g., Tahamtani et al., 2016) due to boredom (Burn, 2017).

5.1.3.1 Process of decision-making

Animal affect is important for coordinating and organising decision-making and behavioural display (Cabanac, 2002; Mendl & Paul, 2020; Nettle & Bateson, 2012). When making a decision, there are four elements that influence an individual's baseline affective state, which impact the choice made (Mendl & Paul, 2020): (1) The individual's current *internal status*, such as hormone levels and body temperature; (2) *Incoming sensory information* from the external environment; (3) *Memories* from the individual's *own past*, in addition to the species evolutionary history ("*evolutionary memory*"); and (4) the individual's *recent history* of reward acquisition and punishment avoidance (core affect history).

After perceiving external stimuli, an individual should match the current situation to similar prior experiences held in memory. This retrieval should be accompanied by information regarding beneficial (rewarding) or disadvantageous (punishing) consequences of actions used previously to determine the best response out of the *affective options* in the current circumstances: approach, avoid or ignore (Mendl &

Paul, 2020; Fig. 4). The selected action (action value) and the predicted outcome of that action (affective prediction) will vary depending on how successful the action has been in previous circumstances. The valuation of an individual is not without error (prediction error) and “mistakes” may increase possibly biased attention to certain stimuli and cause further miscalculations. Once selected, the action will have a physical outcome and an affective outcome. The *physical outcome* is any alteration (or lack thereof) to the physical being of the individual that modify the internal status of the individual through feedback mechanisms. The *affective outcome* is the affective state experienced after the decision has been made. It is influenced by both the expected consequence (affective prediction) and the presence or absence of a reward or punishment. This outcome will update the core affect of the individual and influence future decisions (Fig. 4). It is here that the dimensional core affect model comes in (Fig. 2): if the individual expected a reward (Q2), but was punished instead (Q4), the punishment avoidance system is activated and the individual is more likely to avoid making the same decision in the future. Similarly, if the individual expected punishment (Q3), but was rewarded (Q1), the reward acquisition system is activated, increasing the likelihood of repeating the same decision (Mendl et al., 2010). Therefore, each decision made, and the experienced consequences, influence further decision-making. Hence, development of decision-making can be observed (e.g., changing preference in litter from early development to adulthood, Nicol et al., 2001)

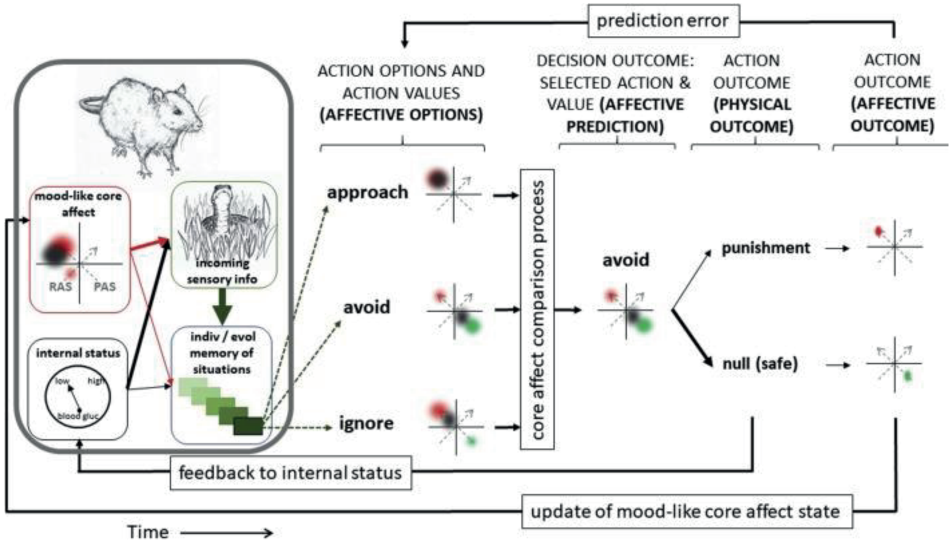

Figure 4. Visual illustration of the role of core affect (valence and arousal) in animal decision making, from Mendl & Paul (2020).

5.1.3.2 Learning and adaptation

While the consequences of decision-making can be beneficial, the decision-making process itself involves some cost. The process requires cognitive processing that may occur extremely rapidly in situations of immediate threat resulting in a habitual response or take some time in non-urgent situations allowing for a more creative response (Knutson & Greer, 2008). The more factors that must be accounted for or that may influence the decision-making process, such as the number of possible actions and the number of prior experiences and associated outcomes upon which to base a choice, the more difficult and costly the decision-making process becomes. Different species vary in their capacity for cognitive decision-making, affected by their information processing- and memory capacity. However, the extent to which animals have a conscious experience when evaluating options (Mendl & Paul, 2020) and engaging in future planning (Raby et al., 2007) is unclear.

The process of decision-making includes both memory retrieval and feedback-loops altering the internal processes of an individual based on rewarding or punishing outcomes (Mendl & Paul, 2020). However, in the aftermath of a decision, an individual will not only learn the consequence of their selected action and its success rate (proto-learning: operant conditioning), but they will also learn how to make further decisions. This process is called “deutero-learning” (Bateson (1972) in Visser, 2003) and can be explained as “learning to learn” (Harlow, 1949). The more experience an individual gathers, the easier it will be to make new decisions and the more complex the decisions can be. Consequently, choice provision could promote cognitive skill in decision-making and organisation of the daily behavioural time budget in addition to physical skills (e.g., dexterity, balance, agility) resulting in an increased sense of confidence and control over the environments. It could also result in more experienced individuals that are better equipped to handle challenges and to predict the likelihood of reward when making further choices (Skinner, 1938; Thorndike, 1898).

According to the Yerkes-Dodson law (Yerkes & Dodson, 1908; Fig. 5), too little cognitive challenge within the environment presents the risk of boredom whereas too great a level of challenge risks chronic stress. How successfully an individual copes with challenges will affect its welfare. Coping can be defined as behavioural responses aiming to reduce negative effects on fitness-related systems caused by aversive stimuli (Wechsler, 1995). If the aversive circumstances can be reduced or removed by the selected coping response, this will lead to a positive alteration in the individual’s core affect (Fig. 2 Q1 & Q2) and will increase the likelihood of choosing this coping response in the future (Wechsler & Lea, 2007). However, if the chosen coping response fails to reduce or remove the aversive stimuli, the corresponding stress may lead to negative alterations in core affect (Fig. 2 Q3 & Q4). If the choices of the individual repeatedly fail to reduce aversive stimuli, they may lead to the build-up of allostatic load (Korte et al., 2005; McEwen, 1998). Allostatic load is the cost of long-term exposure to chronic or repeated challenges that are perceived as stressful by the individual and is generally thought of as the cost of adapting to challenges (Koolhaas et al., 2011; Korte et al., 2005; McEwen, 1998).

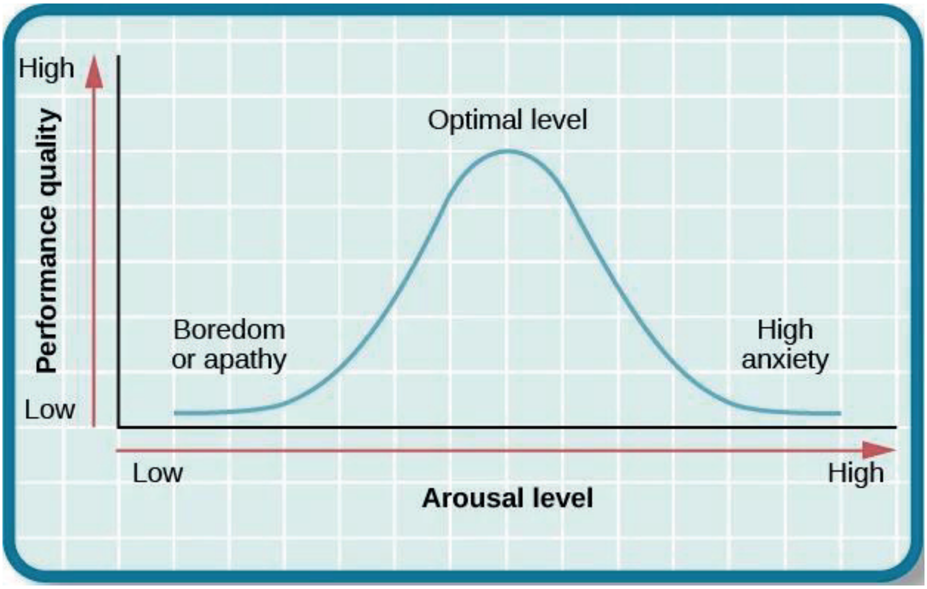

Figure 5. Illustration of the concept of optimal arousal for task performance (Yerkes & Dodson, 1908). At the optimal level of arousal (top of curve), task performance is maximum. At the left end of the curve, there is too little arousal, while on the right side of the curve there is too much arousal. Picture from: https://www.simplypsychology.org/what-is-the-yerkes-dodson-law.html (accessed: 25.08.2023).

Due to environmental conditions in combination with differences in genetics and pre- and post-natal experiences, individuals develop different coping strategies from one another. Consequently, environmental conditions should allow for different approaches to overcoming challenges, which may be facilitated by providing choices. Supporting this hypothesis, Nazar et al. (2022) and Skånberg et al. (2023) reported that laying hen chicks reared with access to multiple choices (multiple types of perches and litter) were less fearful and more adaptable when exposed to a challenge (environmental change) compared to conspecifics housed without multiple choices (a single variant of each resource type).

5.1.3.3 Early choice provision

Although choice provision would likely be beneficial for welfare regardless of when it is provided, the timing of choice provision is likely to affect welfare outcomes. Previously, choice has been reported to have long-lasting effects if provided during early development (Luo et al., 2020; Zocher et al., 2020).

Young animals tend to exhibit greater neural plasticity than adults because their neurological systems are still under development (Gazzano et al., 2008; Sarkar et al., 2019). They are also more affected by exposure to certain stimuli during sensitive periods in early development (Bateson, 1979) that have a lasting impact on their phenotypic development (adaptive plasticity hypothesis; Campderrich et al., 2019; Nettle & Bateson, 2015). Furthermore, postnatal experiences have been reported to result in ‘habitat imprinting’; a strong preference for environmental conditions similar to those experienced in an early life stage (Immelmann, 1975). Mismatch between preferences and actual conditions could therefore result in negative emotional development, causing increased perception of stress and frustration that may be reflected in behavioural displays.

If allostatic load accumulates during development, it might lead to greater and longer-lasting negative consequences than if the build-up occurred in adulthood. Chronic stress, a subjective experience related to physiological status, can negatively affect long-term investment in growth, impacting health and brain development. This can appear as being lateralized neurologically or asymmetric in morphology (Ocklenburg et al., 2016). Therefore, morphological parameters, such as body size, ornamentation and colouring are regarded as honest signals of individual quality. These signals can thus be used as integrative indicators of long-term welfare together with other more invasive measures, such as telomere length (M. Bateson, 2016) and neurogenesis in the hippocampus (Poirier et al., 2019).

Consequently, early development appears to be an especially important period in which to provide optimal environmental conditions, and early access to choice may result in more or longer-lasting benefits than later access (particularly in precocial species). In support of this hypothesis, Campbell et al. (2021) found evidence that laying hens raised in enriched aviaries developed a more “plastic” personality that allowed for greater adaptation to different environments compared to hens raised in unenriched aviaries.

5.1.3.4 Limitations of choice

To summarise, benefits of resource choice provision could improve animal welfare by decreasing boredom, increasing agency, accounting for individual variation, enhancing learning, and maintaining benefits of stimulation by novelty for longer. All of these benefits may result in more resilient and flexible individuals with reduced allostatic load and ability to cope better with challenges.

Although there are many benefits of choice provision, its effects are limited. It will not fully remove all negative affects and may only have incremental influence on welfare if the environmental conditions are already good (ceiling effect). Furthermore, too many choices might be overwhelming and would require prior experience with decision-making to handle, and removal of choices after provision may be perceived as even more negative than the lack of provision in the first place.

It is important to note that severe negative welfare states, such as learned helplessness and depression, negatively impact the learning processes and can be expected to limit the benefits of choice provision. Severe negative core affect might not be “fixed” or “removed” if an animal is subsequently given access to choices, especially if choice provision is the only recovery treatment provided. Yet, choice provision might be used as a preventive measure if provided early in life, enhancing an animal’s ability to avoid such aversive welfare states in the future.

Nevertheless, if early provision of abundant choice is followed by a withdrawal of choices, there is a potential for sensitivity to reward loss (disappointment), whereby a negative incentive contrast is expressed as a drop in motivation to seek sources of reward compared to that of animals that never received the choice. In contrast, positive incentive contrast (elation) may occur if choice provision is unexpectedly added after early life without access to abundant choices (Clarkson et al., 2020; Wendt et al., 2019). Such changes in perceived reward value indicate that responses are influenced by previous experience with or without exposure to the source of reward (Clarkson et al., 2020; Wendt et al., 2019).

5.1.4 Poultry: broilers & laying hens

Environmental enrichment and choices are increasingly used in poultry, making them a good model for studying resource choice, because of extensive scientific knowledge and future need to improve animal welfare for individuals.

Poultry are domesticated avian species that have been bred for egg-, meat- and/or feather production. Normally, the term covers a wide range of species, including ducks and pheasants, but in this thesis the term "poultry" is limited to domestic chickens (*Gallus gallus domesticus*): broiler chickens and laying hens. I chose both types of domestic chicken hybrids as my model animals because they share underlying physiological mechanisms, and are common production animals that have generated public concerns about their welfare. Both broiler chickens and laying hens also have a short generation time, making them a practical model for examining both short- and long-term effects of early choice provision on their behaviour and welfare.

Some welfare concerns differ between broiler chickens and laying hens. For example, keel bone fractures are more prominent in adult laying hens (Petrik et al., 2015) and broiler breeder hens (Gebhardt-Henrich et al., 2018) than in young broilers. In contrast, lameness is a significant welfare problem in broilers (Weeks et al., 2000), especially of fast-growing breeds (Dixon, 2020; Rayner et al., 2020) but is not prominent in laying hens. Nevertheless, there are also welfare issues common between the two types of chickens. One concern is that individual lives have low economic value. Commercial poultry are commonly kept in large flocks consisting of hundreds to thousands of individuals, and the loss of 1% of the animals is considered normal, having relatively little impact on the overall production value. This lack of focus on the individual is also reflected in the consideration of animal welfare: it is the welfare of the flock rather than each individual that is emphasised. Furthermore, due to large group sizes, it can be difficult to detect unfit individuals, especially if the problem is not immediately visible. Although choice provision cannot correct these issues, it could still improve the welfare of individual birds by contributing opportunities for positive experiences.

Perches and litter have been found to be important resources for poultry, with litter being important for both broilers and laying hens, and perches being especially important for laying hens (e.g., Aerni et al., 2005; Gunnarsson et al., 2000). Skånberg et al. (2021) reported that laying hen chicks spent most of their first four weeks of life on litter, followed by sitting on a perch, foraging, resting, preening and dustbathing (in order of decreasing % of time spent). The chicks displayed individual differences in their use of different resources (perches and litters) already during their first week. Gunnarsson et al. (2000) reported that laying hen chicks raised without perches displayed decreased spatial skills required for three-dimensional movement compared to conspecifics that were reared with perches. Aerni et al. (2005) found that laying hens reared with access to litter material in their first four weeks of their life had lower food conversion and mortality rates, in addition to increased egg weight and egg mass, compared to conspecifics reared without litter. Similarly, Johnsen et al. (1998) found that laying hen chicks reared on either sand or straw during their first four weeks of life, had better plumage scores and displayed less feather pecking and less cannibalism when older compared to birds reared on wire netting. Consequently, it could be beneficial to provide choices of different perch- and litter types to poultry and it would not be difficult to implement choice provision in commercial production systems. Research on poultry has also confirmed that different personalities exist in domestic chickens (Garnham & Løvlie, 2018), suggesting that large flocks could benefit from choice provision to accommodate individual differences. Furthermore, hens raised in more complex environments have been reported to be less fearful as adults (Brantsæter et al., 2016), to display better short-term spatial memory and to adapt more quickly to environmental change (Campbell et al., 2021) compared to hens reared in simpler environments, supporting the hypothesis that early choice provision could be beneficial for poultry.

The breeds used as model animals in this thesis project were Ross 308 (broiler chickens) and Bovan Robust (laying hens), both hatched at commercial hatcheries. Although I would very much like to generalise my results to poultry in general, or even other species, the results are specific to these hybrids. The Ross 308 (Fig. 6a) is a fast-growing breed used for meat production. In Norway, these chickens are generally kept in one flock per farm comprising both females and males until approximately 32 days of age when they are transferred to a processing plant for slaughter. They are required to have floor litter (with wood-shavings being the most common litter type), access to feed and water, a minimum of 6 hours of daily darkness, and a density no higher than 36 kg/m^2. There is current debate in Norway regarding use of the Ross 308 as it is more cost-efficient for the farmer to grow fast-growing broilers whereas slower-growing breeds are reported to have better welfare (Dixon, 2020; Forseth et al., 2023; Rayner et al., 2020).

Bovan Robust (Fig. 6b) is a breed selected for egg production and is specifically suited for cage-free production systems. Currently in Norway, chicks are sexed at the hatchery and only the females are kept. No beak treatment is permitted. The pullets are typically reared in tiered aviary systems with a maximum of four levels. For the first 3-5 weeks of life, they are confined to one of the lower aviary tiers before the compartments are opened giving them access to litter on the floor. Prior to the onset of lay (around 18 weeks of age), they are moved to an aviary facility specialised for egg production, where they are housed in flocks with a maximum of 7,500 hens. Here they are required to have access to perches (minimum 15 cm/hen), nests (minimum 1 m^2/120 hens) and litter material (minimum 250 cm^2/hen covering $^1/_3$ of the floor area) in addition to feed and water.

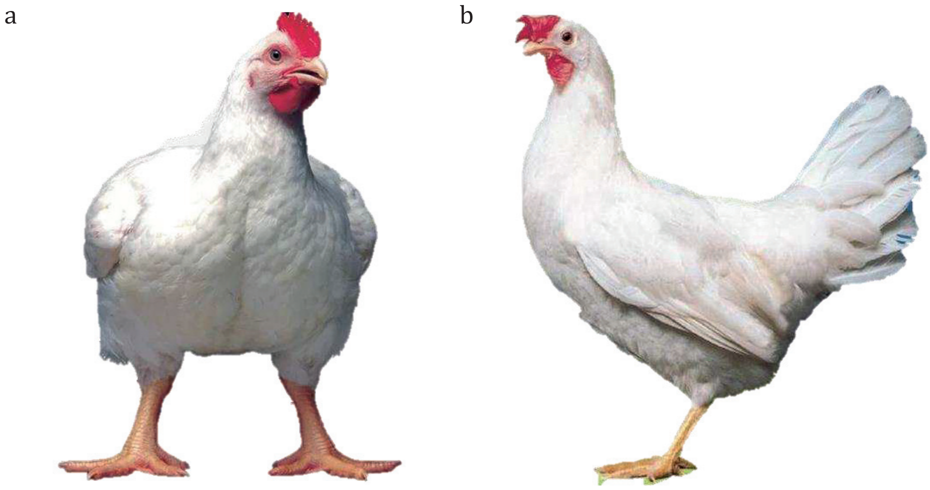

Figure 6. Chicken breeds. a) Ross 308: broiler chicken bred for meat production. Photo: Aviagen. b) Bovan Robust: laying hen bred for cage-free egg production. Photo: Swefarm.

5.1.5 Aim

Because of complex mechanisms underlying choice (section 5.1.3.4), the general objective of this thesis was to develop knowledge on the value of resource choice as a form of environmental enrichment that would provide opportunities for positive experiences, accommodate individual differences, contribute to stress resilience and be relatively easy to implement in commercial systems. More precisely, I aimed to examine the effects of resource choice on poultry behaviour and associated positive and negative welfare indicators. This aim was achieved by conducting two studies leading to three scientific articles:

1. A field study on broiler chickens investigating their behavioural responses to resource choice and to the novelty associated with refreshment of the choices (paper **I**).

2. An experimental study with laying hens examining long-term effects of offering resource choices during different phases of rearing (papers **II** and **III**).

The hypotheses pursued in the three papers were:

1. Broiler chickens would prefer multiple litter types, because they have different functions, in turn stimulating different kinds of behaviour. Refreshment is needed to maintain interest and desired activities.

2. Provision of multiple vs single resource types would increase incidences of positive behaviours and decrease negative behaviours, improving laying hen welfare in both the short-and long-term.

3. Comb morphology would be affected by the degree of resource choice experienced during rearing (weeks 1-15), due to being a condition-dependent signal.

5.2 Materials and Methods

This section gives an overview of the different materials and methods used in the two studies. Details can be found in papers **I-III**.

5.2.1 Animals and housing conditions

In the studies presented in this thesis, all animals were non-beak-trimmed, vaccinated and of normal health. The birds were hatched at local hatcheries and delivered as day-old chicks. All birds were given *ad libitum* access to feed and water and had litter covering the floor. Light intensity, light cycles and the room temperature were adjusted according to breeder recommendation for commercial production. In paper **I**, two consecutive flocks of mixed-sex Ross 308 chickens (each flock comprising approximately 17,000 chicks) with peat-litter were studied under commercial conditions at a farm in Eastern Norway for four weeks (1-4 weeks of age). Within their first week, they were given access to seven different litter types in two different locations in the house. Perches (boxes) and foraging material (silage) were spread around the house when the birds were two weeks of age. For papers **II** and **III**, 365 female Bovan Robust (Swefarm, SE) pullets were studied in an experimental set-up for 27 weeks (0-27 weeks of age). The laying hen chicks were pseudo-randomly distributed among 16 pens (22 or 23 birds/pen) within one room at a research facility in Uppsala, SE, (Swedish Livestock Research Centre). All pens had the same set-up of four perches, four litter trays, a round feeder, a row of water nipples, and a heat lamp (present during the first four weeks only), and were managed in the same way (same feed, water, lighting, temperature, etc.). Visual communication between pens was limited by wrapping pen walls with heavy brown paper. All groups were kept stable throughout the experiment.

5.2.2 Experimental design

In the field study, the two broiler flocks were given access to a pair of seven adjacent 1 m^2-litter trays, placed at each end of the chicken house, from the first weeks of age until they were 34 days old. In each set of trays, each tray contained a different litter type (Fig. 7). The seven litter materials were peat (control), peat diluted by mixing it with wood shavings, plain wood shavings, finely-chopped pine bark, oat straw pellets, coarsely-crushed rape straw, and finely-ground rape straw, placed in a random order in each set of trays. We collected data once per week for four weeks. Halfway through each weekly observation (after 31.5 min), 5 L of fresh material was added to each litter tray, enabling comparison of responses towards the "used" vs fresh" litter materials.

Figure 7. Broiler chickens exploring parts of a litter buffet offering different litter types (Photo: Ruth C. Newberry).

In the experimental study, the laying hen pullets were reared in pens with either one or four litter- and perch types for 15 weeks before being moved to standard adult laying hen pens. In weeks 1-4 (Period 1), half of the pullets were kept in a less complex environment with four perches and four litter trays of the same type ("single-choice"; Fig. 8 a & c), while the remaining pullets were reared in environments with four different perch- and litter types ("multi-choice"; Fig. 8 b & d). The four perch types were a pine plank, a thick rope (three braided horse leads), a round rubber perch and a flat wire mesh perch in a spruce frame. The litter types were fine-grained sand, wood shavings, straw and peat. One of these litter types also covered the pen floor (balanced across pens). In Single-choice pens (n = 8), one perch type was paired with one litter type, resulting in four combinations that were balanced across pens, where our focus was on the effects of having less choice regardless of which type of perch and litter was present in each pen.

Multi-choice pens (n=8) had one of each of the four different perch- and litter types, with the relative location of each type balanced across pens. Here, we focussed on the effects of having more choice and not on the relative location of each perch- and litter type within the pen. After Period 1, half of the pens were switched to the opposite treatment (week 5-15: Period 2; 4 pens/treatment). From 16-27 weeks (Period 3), all groups were kept in similar adult laying hen pens with novel perch- and litter types (Fig. 8e).

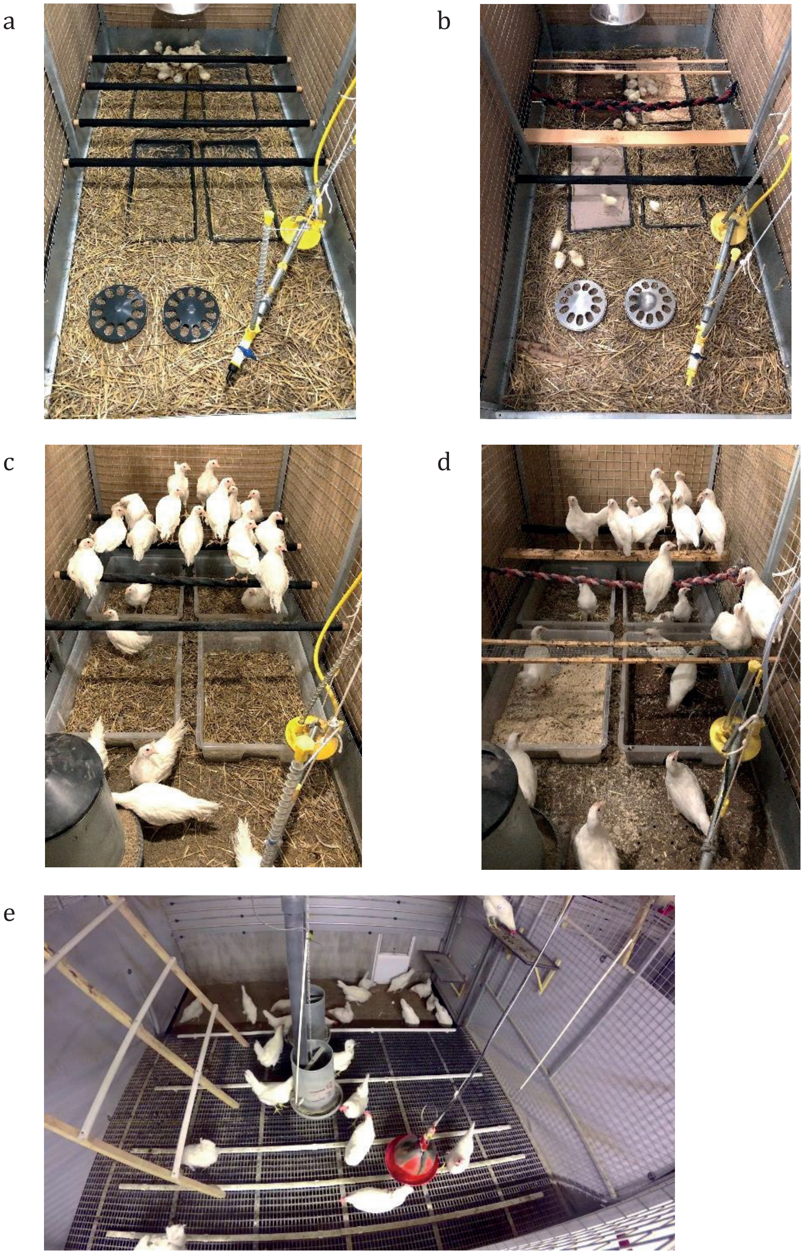

Figure 8. Examples of pen set up in experimental study (more examples in paper **II**). First row: single choice treatment (a) and multi-choice treatment (b) in period 1 (week 1-4). Second row: single-choice treatment (c) and multi-choice treatment (d) in period 2 (week 5-15). Third row: adult pens (e), similar for all groups (Photos: Lena Skånberg).

5.2.3 Data collection

Following well-established methods of data collection (Martin & Bateson, 2007) we used instantaneous scans to collect data on the number of birds present in different areas at that moment. These instantaneous scans were always followed by a longer scan to collect data on the frequencies or occurrence of behavioural events (paper **I**: 15 s 1-0 scan; paper **II**: 3 min scan for counts and 1-0 scores).

For paper **I**, we observed the total number of chickens present in each litter tray (Fig. 9a) followed by the number of chickens observed performing different litter-directed behaviours (ground scratching, dustbathing and lying resting) in each tray, to assess the use of the different resource choices (here litter types). As we had two sets of seven adjacent litter trays, one on each end of the barn, we summed the results per litter type per observation period. In the paper, results are expressed as the number of birds present or engaged in a particular behaviour in each litter type over the four weeks of observation. The particle size distribution was also measured for both used and fresh materials using sieve analysis (Fig. 9b).

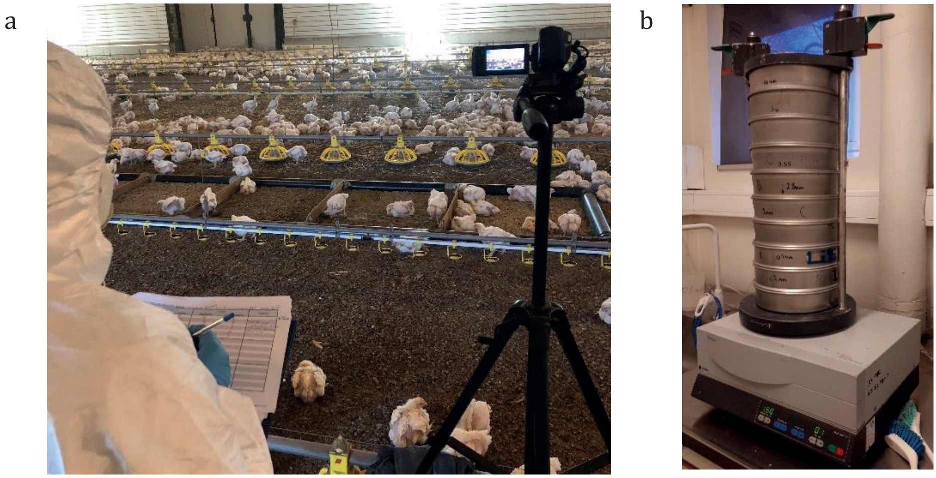

Figure 9. Data collection for field study (paper I). a) Observation of litter-directed behaviours in a litter buffet offering several different types of litters (Photo: Guro Vasdal). b) Set-up of sieve-analysis for litter particle size distribution (Photo: RV Holt).

In paper **II**, we collected behavioural frequencies associated with positive (play and dustbathing) vs negative affective states (vigilance, aggressive pecking and severe feather pecking) to examine long-term effects of the single-choice vs. multi-choice conditions (Fig. 10a). Instantaneous scans were used to determine the number of vigilant birds before the group-level frequencies of the remaining behaviours were determined in a 3-minute observation. Inter-observer concordance was monitored regularly and maintained at 90% or higher using trial observations and frequent discussions. Because of low numbers with many zeros, some results were analysed as binomial data (proportion of birds performing each behaviour per scan per pen: play and dustbathing). Vigilance was analysed as number of birds per scan per pen, while aggressive pecks and severe feather pecking was analysed as frequency of occurrence per scan per pen. Morphological measurements, including body weight, feather damage score (Fig. 10b) and comb score were also collected for paper **II**. Scores (feather damage and comb damage) were analysed as ordinal variables, while body weight was analysed as a continuous linear variable.

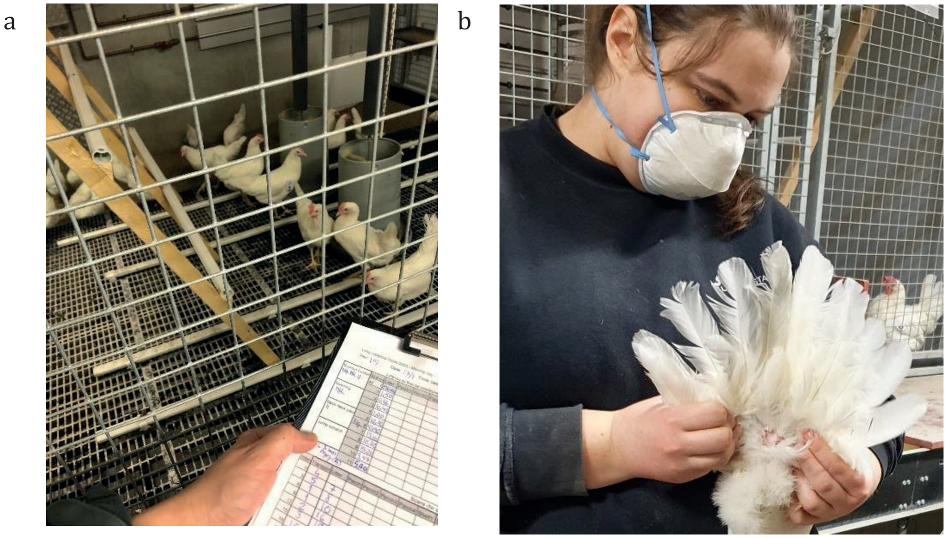

Figure 10. Data collection from experimental study (paper **II**). a) Behavioural data collection on adult hens (week 16; photo: Lena Skånberg). b) Collection of health data, here scoring of tail feather damage (week 27; photo: Yezica Norling).

For paper **III**, we recorded the comb laterality of the hens (e.g., side of the head on which the comb hung) during week 27 and photographed their combs (Fig. 11a). A bespoke image analysis programme was then used to capture comb area and shape complexity (rugosity) from the photographs of 100 hens (n=6-7 hens/pen; Fig. 11b). Inter-observer concordance was calculated by analysing the same 16 pictures (1 picture per pen; randomly selected out of the 100-pictures sampled) using the custom-made picture analysis program (intraclass correlation: 0.88). Results for these 16 pictures from the bespoke image-analysis programme were also compared with those from a recognised image-analysis program (ImageJ; Schindelin et al., 2015), which gave an intraclass correlation of 0.93.

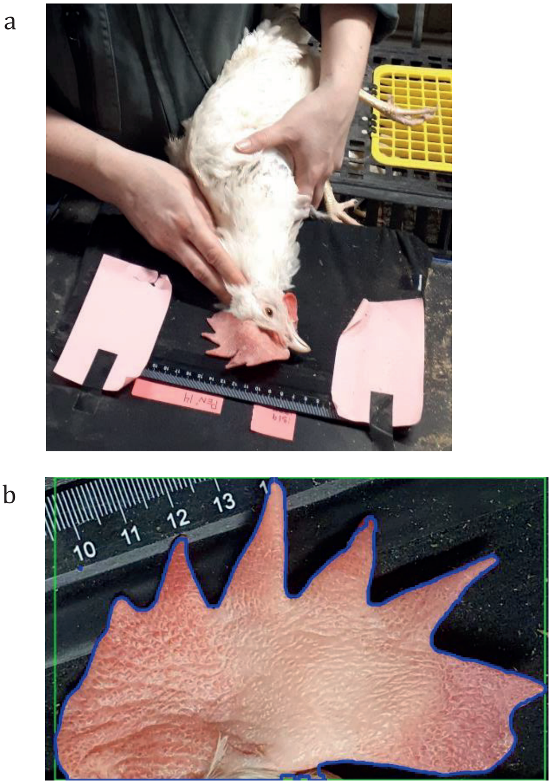

Figure 11. Collection of comb data from experimental study (week 27 photos: RV Holt). a) Placement of live chicken for "mugshot"/comb picture. b) Comb characteristics as analysed by a custom-made image analysis programme.

5.2.4 Data analysis

All statistical analyses were conducted using the statistical environment R (R Core Team, 2023). The statistical models used depended on the variable type of the response, but usually included a random factor to account for groups (flock or pen).

5.2.5 Ethical note

In the field study (paper **I**), all procedures complied with the Norwegian ethical standards for live animal research and no ethical approval was required. All experimental procedures in the experimental study (paper **II** and **III**) were approved by the Uppsala Animal Experiment Ethics Board Number 5.8.18-11549/2017.

5.2.6 Data availability

All of the raw data and r codes used for paper I-III are available online. The URLs to the files, divided by paper, are listed below.

Paper **I**: https://github.com/RVHolt/BuffetOfLitters.git
Paper **II**: https://github.com/RVHolt/LongtermChoice.git
Paper **III**: https://github.com/RVHolt/ChoiceCombs.git

5.3 Results

5.3.1 Paper I

The aim of this paper was to investigate the choice of different litter options for different behaviours. We predicted that all litter options would be utilised, but certain options would be more preferred than others for specific behaviours. Because it was necessary to refresh the litter trays weekly to maintain access to the different litter types, we also investigated the hypothesis that the “freshness” of the litter materials (i.e. their state of novelty, cleanliness and intactness) would affect their use by chickens for different behaviours.

We found that fresh litter attracted many more birds than used litter for active behaviours such as ground scratching and dustbathing. Freshness was not selected for inactive “lying resting” behaviour, and as this behaviour accounted for a majority of the behavioural time budget, the overall probability of selecting fresh vs used litter only approached significance.

As expected, different litter types were generally preferred for different behaviours. Ground scratching occurred mostly in peat and mixed peat. Dustbathing occurred most often in mixed peat, peat and fine rape straw when the litters were fresh, but fine rape straw attracted the most birds when used; Wood shavings were used the most for lying resting and in total, followed by mixed peat and fine rape straw.

Based on these results, we concluded that broiler chickens preferred different resource choices for different behaviours and that providing multiple litter choices would help birds to satisfy different behavioural motivations. Furthermore, as fresh litter was preferred over used material for active behaviours, we concluded that refreshment of the litter materials would be necessary to maintain interest over longer periods.

5.3.2 Paper II

The aim of paper **II** was to examine the effects of providing resource choice during development on short- and long-term measures of behaviour and health in laying hens. This was of practical interest because laying hens are exposed to environmental changes during commercial rearing and transfer to laying facilities (Colson et al., 2008). Although it is recommended to match the rearing and adult housing conditions (Janczak & Riber, 2015), this is not always feasible. There is thus a need for methods of increasing the stress resilience and adaptability of pullets, which may be achieved through early experience with multiple resource types. Resource choices were presented in the form of different perch- and litter types, as previous literature has demonstrated the importance of early access to these resources for normal pullet development (e.g., Aerni et al., 2005; Gunnarsson et al., 2000). Furthermore, as novelty was found to be important for active behaviours in paper **I**, the litter materials were refreshed at least once weekly during development to maintain interest. We expected behaviours associated with positive affective states, here play and dustbathing, to increase in frequency and behaviours associated with negative affective states including vigilance, aggressive pecking and severe feather pecking, to decrease in frequency with exposure to resource choice. We also expected resource choice to promote fitness-related changes, including higher body weight, less feather damage and comb damage, and lower mortality.

Our results showed that during the first four weeks of life, laying hen pullets played more and displayed lower levels of dustbathing and severe feather pecking if they did than did not have access to resource choice during this period. They also dustbathed more and were less likely to perform severe feather pecking if they had access to resource choice later in development (week 5-15). After being moved to the standard adult environment (week 16-27), hens that experienced resource choice early in development (week 1-4) displayed lower rates of aggressive pecking than hens without choice during this period, while hens that experienced resource choice later in development (week 5-15) displayed more dustbathing. We also found that adult hens housed with resource choice later in development (week 5-15) had higher body weights and better plumage condition than hens without this access to choice. We found no effect of resource choice during either developmental period on vigilance, comb damage, or mortality of the birds.

Based on the results of this study, we concluded that exposure to environmental choice during development had fitness-enhancing effects on laying hen behaviour, some of which had long-lasting effects into adulthood. Although we did not find evidence that choice provision had more benefits during a particular developmental period (week 1-4 or week 5-15), providing environmental choice throughout development resulted in the best welfare outcomes for laying hens.

5.3.3 Paper III

As we found behavioural and morphological differences between hens housed with and without resource choice (paper **II**), we predicted that hens housed with resource choice during development would have larger, more complex and left-side biased combs when adult (week 27), reflecting lower allostatic load, than hens housed without resource choice during development. The predicted laterality was based on a possible bias in head posture or head movements associated with greater right eye and/or ear use and left-brain hemispheric dominance. Contrary to our predictions, we did not find any evidence that comb development differed between treatments. However, we detected an overall right-side bias in comb laterality and that birds with right-lopping combs (“righties”) had longer perimeters than birds with left-lopping combs (“lefties”; Fig. 12). "Righties” with larger combs also tended to be heavier and to have less comb damage than “righties” with smaller combs. “Lefties” with larger combs were also heavier and had less feather damage than smaller-combed “lefties”.

Based on these results, we concluded that the contrast between treatments on the positive end of the welfare spectrum was insufficient to alter the trajectory of comb development.

Figure 12. Examples of comb lateralisation in adult laying hens (week 27). Left: Individual with left lopped comb (“lefty”). Right: Individual with right lopped comb (“righty”). Photos: Yezica Norling.

5.4 Discussion

This thesis examined the effects of resource choice on the welfare of poultry (broiler chickens and laying hens), utilising both positive and negative behavioural indicators. We selected behavioural indicators predicted to be sensitive to changes in environmental conditions. Physical traits were included to further assess long-term fitness consequences and complement the behavioural results. Overall, our results show that the provision of a more complex environment with possibilities to interact with several attractive resource variants has clear short- and long-term benefits for the behavioural development and welfare of poultry.

5.4.1 Behavioural development and positive welfare

We selected behavioural indicators associated with both positive- and negative affective states in chickens. The positive-valenced behaviours examined were play behaviour, dustbathing, and exploration. The negative-valenced behaviours were vigilance, aggressive pecking, and severe feather pecking. Physical indicators of welfare associated with these behaviours were also examined, including body weight, feather damage, comb damage and comb morphology traits (size, shape complexity and laterality).

Play behaviour usually occurs in the absence of fear and discomfort. Play is hypothesized to be important for several developmental aspects, such as improving motor-, cognitive- and social skills, in addition to learning how to cope with unexpected events and environmental challenges (Špinka et al., 2001). In paper **II**, we report that play behaviour occurs more during early development (week 1-4) than when older. In accordance with our results, play has previously been reported to decline with increasing age in poultry (e.g., Dawson & Siegel, 1967; Vasdal et al., 2019) and other species (Bekoff & Allen, 1998). Dustbathing has been recognised as a comfort behaviour, assumed to be positively valenced as it enhances plumage quality (Olsson & Keeling, 2005; van Liere, 1992). Furthermore, poultry are willing to work for access to dustbathing substrates (Widowski & Duncan, 2000). In paper **II**, we found that dustbathing tended to increase as the birds grew older, probably due to the plumage requiring more extensive care as the number of feathers increases with age. Ground scratching is a motor pattern commonly used in foraging and exploration. This behaviour was studied in paper **I**, where we report that the frequency of ground scratching decreased with increasing age in broilers, similar to the frequency of play behaviour in paper **II**. This is likely a consequence of increased mobility issues in fast growing broilers as they become older (Rayner et al., 2020).

Vigilance, useful for detecting potentially dangerous stimuli (Newberry et al., 2001; Zidar & Løvlie, 2012) and therefore thought to be a negatively-valenced behaviour associated with fear or anxiety (Campbell et al., 2019), has previously been reported to increase with age (Newberry et al., 2001). This is consistent with the reported difference in vigilance between early (week 1-4) and later development (week 5-15) in paper **II**. The following decline in vigilance from late development to adulthood (week 16-27) might be a result of the spacious adult environment allowing the birds to avoid perceived danger to a greater extent. Aggressive pecking and severe feather pecking are associated with negative welfare, as they can cause stress and painful wounds for the receiver (Gentle & Hunter, 1991). Aggressive pecking tends to increase as poultry approach sexual maturity (McKeegan & Savory, 1998), which is consistent with our findings presented in paper **II**. Severe feather pecking has been hypothesised to result from redirection of foraging behaviour to pecking at conspecifics. However, Newberry et al. (2007) found no clear evidence for this when tracking the behaviour of individual laying hens over time from 3-15 to 17-37 weeks of age. We observed the highest frequency of severe feather pecking in weeks 5-15 (paper **II**), the period with the lowest accessibility of resources when considering the increasing body size of individuals with increasing age. Likely the increased floor and perch space per bird and greater complexity of the adult pens allowed for better avoidance of severe feather pecking. The increased feather damage scores in the middle of the study period (week 5-15), which remained present in adulthood (week 16-27), were likely a result of the higher frequency of severe feather pecking in the middle age period.

Although a primitive version of the comb is already present at the time of hatching, a chicken comb is not fully developed until after the birds reach sexual maturity. Consequently, at the time we collected data on comb characteristics (week 27) for paper **III**, the combs of our laying hens were assumed to be fully developed. Combs are known as condition dependent signals in both chickens (Bakovic et al., 2022) and their progenitors (Red jungle fowl: Zuk et al., 1990), where the quality normally declines under adverse environmental conditions. Characteristics of chicken combs have been associated with fitness-related measures such as body size (Tufvesson et al., 1999) and fecundity (Wright et al., 2012), in addition to being an important indicator of social status (O'Connor et al., 2011). Although we found body weight to be affected by resource choice in paper **II**, the resource choice treatments did not have significant effects on comb size, shape complexity or comb laterality in paper **III**. This could be because there were relatively small differences between treatments, as also suggested by Campbell et al. (2021) when they found no differences between simpler and more complex rearing conditions on personality traits in laying hens. If the simpler environment with less choice is sufficiently good, the incremental benefits of more choice on the positive end of the scale may not be reflected in differences in morphological traits, such as those expressing comb quality.

It is important to note that none of the environments included in this project could be described as adverse or barren. This was a deliberate decision as many studies have previously explored the effects of barren/adverse vs. enriched/complex environments (Jacobs et al., 2023), often mainly providing more evidence for the need for what some countries now consider fundamental provisions for basic animal welfare (based on the Five Freedoms concept). In the current work, we wanted to move a step further and include a focus on positive animal welfare, providing suggestions on how to move towards "a life worth living", or even "a good life" for poultry with an increase in environmental complexity.

5.4.1.1 Choice of enrichments

In many countries, farmers are required by law (e.g. Council Directive 98/58/EC Concerning the Protection of Animals Kept for Farming Purposes, 1998; Lov Om Dyrevelferd, 2009) to provide perches and litter for adult hens and litter for broilers, although regulations concerning pullets are lagging. This is despite knowledge that the absence of these resources during rearing has negative welfare consequences (e.g., Aerni et al., 2005; Brantsæter et al., 2016; Campbell et al., 2021; Gunnarsson et al., 2000; Johnsen et al., 1998). In the present work, all birds received litter (paper **I**), or litter and perches (papers **II** and **III**) as the basic control condition, allowing us to focus on provision of choice of these materials as the enrichment. Perches and litter substrates of different types were chosen for two main reasons: Firstly, litter and perches satisfy behavioural needs in poultry, and were expected to reduce negative behaviours and stimulate positive activities such as play, exploration and dustbathing. These behaviours are expected to occur when individuals feel safe and positively engaged. Secondly, we wanted to work with choice of resources that could be feasible to use in commercial production. Consequently, we used commercially relevant products.

Skånberg et al. (2021) reported that laying hen chicks preferred a relatively wide rope-perch in the first week after hatching and mostly used it for resting. When preening, however, laying hen chicks preferred flat, sturdy perches over the more unstable rope-perches. They were also more likely to perform successful landings on wider perches (both rope- and stable perches) than narrow ones. Consequently, we decided to include wide perches in our experimental study, including a rope-perch and stable perches of different shapes. Paper **I** revealed that broiler chickens preferred peat, wood shavings and peat mixed with wood shavings for ground scratching, but particle size had no effect on frequency of ground scratching in the different litters. They were, however, much more interested in ground scratching when materials were ‘fresh’. Dustbathing also occurred more frequently when litters were fresh and broilers showed a preference for litters with smaller particle sizes, such as peat, peat mixed with wood shavings and finely-cut straw. When litters were “used”, however, dustbathing occurred more frequently in finely-cut straw, which was beneficial if clumping was minimal. The wood shavings were preferred for resting. The birds also preferred to rest in “used” litters with fewer small particles and in fresh litter with fewer large particles. These findings highlight the importance of providing materials with preferred qualities to increase levels of positive and desired behaviours. Furthermore, these results support the provision of multiple types of the same resource that compliment one another, as different qualities are preferred for different behaviours.

Although we decided on choice of perches and litter as enrichments, other variants of other resources could be used instead or in addition to these to improve life quality of poultry. Platforms, shelters, feed, feed mixed in litter and different heights of nest boxes, are all perceived as attractive resources for poultry. Scattering of high value feed items (such as different types of insects), have been reported to increase activity and foraging if frequently provided (Ipema et al., 2020; Pichova et al., 2016). Barriers (e.g., vertical screens or low dividers) have been reported to increase resting (Newberry & Shackleton, 1997), decrease disturbances when resting (Ventura et al., 2012), and increase perching behaviour (Ventura et al., 2012), compared to when barriers were absent. Forslind et al. (2021) also reported that the presence of platforms decreased the level of physical disturbance experienced by broiler chickens when resting compared to broilers resting in open areas. Similarly, Forslind et al. (2022) reported that birds raised with artificial dark brooders (a type of heated shelter) were less exposed to disturbance when resting and were more likely to complete spatial learning tasks than broilers reared without artificial brooders. Regardless of the enrichments provided, they should stimulate positive valenced behaviours and affective states, and if more than one variant of each type is to be provided, they should complement each other to facilitate behavioural diversity.

5.4.2 Novelty and familiarisation

In paper **I**, ground scratching and dustbathing were much more frequent after fresh litter material had been added to replenish each litter box compared to when only “used” material was available. This is consistent with other findings (in pigs: Modlinska et al., 2019; in rats: Trickett et al., 2009), where new resources increase initial exploration frequency. Enrichments are primarily effective because they add stimuli to an otherwise monotonous and predictable environment. Another attractive feature of enrichments is the opportunity to destroy them (van De Weerd et al., 2003). This presumably allows animals to learn about characteristics of the item as well as allowing them to develop skill in manipulating them. While individuals tend to show initial fear towards novel environments and objects, thus tending to prefer familiar situations and places (Wiepkema & Koolhaas, 1993), some degree of novelty is necessary to motivate exploratory behaviour (e.g., in rats: Hughes, 1968) Nevertheless, familiarisation to materials that are found to be non-threatening occurs rapidly (e.g., in pigs: Guy et al., 2013) and this can greatly affect the frequency of their utilisation as very familiar materials are not as stimulating as somewhat novel materials or familiar materials in new locations (in domestic fowl: Newberry, 1999; in pigs: Wood-Gush & Vestergaard, 1991). The results of paper **I**, indicate that renewing familiar resource choices enhances their attractiveness.

“Refreshment” or “renewal” of resources by altering or adding more of the same resource is one of several methods that may be used to slow down familiarisation with enrichments. This method is practical for resources such as litter (papers **I-III**), although entailing the cost of using a larger quantum of each resource. A second method is making resources available only for a short time through rotation of resources (in pigs: Gifford et al., 2007) or frequently providing new types of resource, defined by Skånberg et al. (2023) as change as opposed to choice. However, there is some indication that provision of a single type of resource at a time might not be as effective as simultaneously providing resource choice (BenSassi et al., 2019; Skånberg et al., 2023; Vas et al., 2023).

5.4.3 Spatial requirements

Based on a modelling approach, the 2023 EFSA report (Nielsen et al., 2023) has recently recommended that all poultry be kept at densities no greater than 11 kg/m^2, which is far lower than is typical today (e.g. EU legislation: max. 42 kg/m^2 for broilers). BenSassi et al. (2019) found fewer indicators of negative welfare in broiler chickens when kept in environments with a greater floor space. Furthermore, Vas et al. (2023) reported higher levels of locomotory play when increasing floor space per bird by reducing stocking density from 29.6 to 18.1 kg/m^2. To express positive behaviours, such as play and exploration, enough space is needed, especially if individuals would prefer to be synchronous in their behaviour.

A greater space allowance does not only allow for more locomotion and freedom of movement but is also likely to provide animals with an experience of greater control over their environment (allowing for easier environmental orientation and variable personal space). Provision of resource choice will require some space, and it is therefore important that enrichments are biologically relevant. If not, they may mostly be taking up space that increases the animal density, leading to poorer animal welfare. Fortunately, Norwegian poultry farmers who provided enrichments for broilers offered an increased floor space as well (Vas et al., 2023), and greater space also stimulated greater use of the enrichments, as also observed in pigs (Jensen et al., 2010).

The stocking densities in the current work were well below the maximum permitted densities in commercial production. In the field study, the broiler density was about 34 kg/m^2 as opposed to the 36 kg/m^2 Norwegian legislated limit. In the experimental study, the density at floor level (excluding perches) was 1.05 kg/m^2 at the end of early development (week 4), 8.61 kg/m^2 at the end of rearing (week 15), and 2.70 kg/m^2 at the end of the experiment (week 27; in the adult laying house) as opposed to the 14.23 kg/m^2 EU legislated limit. Our birds should therefore have had sufficient space to express positive behaviours and fully utilise enrichments. However, the movement of the birds was sometimes observed to be interrupted when reaching the sides of the litter trays, in such a way that it might have limited locomotory play, especially in the period from week 5-15.

5.4.4 Implications

This thesis provides a compelling argument for the provision of multiple versions of similar resource types, referred to as "resource choice", in poultry. It provides suggestions on how to improve the welfare of individual broiler chickens (paper **I**) and laying hens (paper **II**), both common production species kept in large groups and at relatively high densities. Proposed benefits of choice provision include accommodating individual difference, increased learning (proto- and deutero), and better behavioural adaptability, resulting in more competent and resilient individuals. Our findings contribute to the basic understanding of outcomes of providing choice as enrichment in animal environments (papers **I-III**), including how choice provision during early life affects the trajectory of behavioural- (papers **I** and **II**) and morphological development (papers **II** and **III**). Moreover, the utilisation of hypothesised associations between affective states and behavioural displays (papers **I** and **II**) was useful for interpreting the behavioural results.

Practical considerations when providing resource choice include the importance of maintaining interest in the provided enrichments, and to ensure individuals have enough space to use the enrichments. Providing ample space with biologically relevant enrichments, and frequently changing how the enrichments were presented (e.g. through refreshment), gave beneficial results (papers **I** and **II**). One should also consider the continuity of offering a complex environment from early to later age, as transition from enriched to more barren environments in later phases of the production may cause stress-related behavioural problems and have a negative impact on welfare although not detected in the current work (paper **II**). Our results combined with those of Skånberg et al, (2023) suggest that continuous access to resource choice, while using refreshment of litter materials in the same relative locations to maintain engagement, will likely lead to the most beneficial effects of resource choice on poultry behaviour and welfare.

For commercial conditions, one also needs to consider economic and management factors that limit the use of certain resources. For instance, these include purchase cost of the different resources, farmer effort (e.g., repeated distribution, time allowance, cleaning), a requirement for added storage space for keeping the different materials, and hygienic requirements for maintaining this storage space. A cost-benefit analysis, comparing the animal welfare benefits and these economic costs, should be examined. Nevertheless, there is a need to emphasise development of more varied environments in future commercial conditions, which include areas with different functions to move between. This can facilitate dispersion of individuals, which may reduce behavioural problems such as aggression and severe feather pecking, and stimulate positive behaviours. Larger flock sizes imply a need for more floor space and vertical space, but providing platforms and perches at different levels is likely to give additional positive effects on welfare. Since building costs are great in some countries, Norway included, implementing suitable outdoor areas for poultry should also be considered. Not only is outdoor space cheaper, but it is also easier to provide a more complex environment with less management than by continuously adding new stimuli to an indoor environment.

5.5 Identified gaps for future study

Overall, the papers included in this thesis provide a foundation for the exploration of resource choice as an enrichment in captive environments. Knowledge gaps remaining for future research include evaluating whether species and breeds within species differ in their response to resource choice (see section 5.1.4), as well as the best way to present choices and maintain interest (see section 5.4.2). Adding an element of cognitive challenge for gaining access to resource choice could be of interest (cognitive enrichment) as long as the level of challenge falls within the levels needed to promote flow (optimal arousal for learning; see sections 5.1.1 and 5.1.3.2) rather exerting undue stress. For example, this might be achieved by applying the concept of contrafreeloading (the willingness to work for a reward even though the same reward can be accessed without effort; Jensen, 1963), which offers cognitive and physical challenge to access familiar resources (e.g., in grizzly bears McGowan et al., 2010).

In addition to consideration of individual morphological responses to resource choice (paper **III**), future studies should examine individual differences in behavioural responses to choice, and their association with behavioural flexibility and coping mechanisms. While the current work controlled for group size, it is also relevant to take stocking density and enclosure size into account in such studies, as these three factors can all play a role in welfare outcomes (Estevez et al., 2003; 2007) and may interact with responses to resource choice.

Some limitations of the current work that require further study include the extent to which results are dependent on the specific choices given vs. choice itself. In the experimental study (paper **II**), we controlled for providing different resource variants by balancing them across groups in the single-choice treatment and across location in the multi-choice treatment. However, results could vary if different sets of resource variants were given. For example, the benefit of the multi-choice treatment on plumage condition (paper **II**) may have been due to peat alone, which is a highly attractive dustbathing material for chickens (paper **I**). Moreover, we cannot tell how the benefits of our multi-choice treatment (paper **II**) were influenced by the choice of litter, perches or both, and we have not investigated if spatial or temporal diversity of litter and perch choices could be useful, as opposed to their continual presence in a specific location. It is possible that having a more dynamic, frequently refreshed choice of litter materials was balanced by having continuous access to a predictable set of perch options, allowing the birds to retreat to familiar, elevated locations to rest and feel secure.

It is also unclear whether a mixture of resources could produce as beneficial outcomes as providing each choice separately as suggested by the attractiveness of peat mixed with wood-shavings, which was almost as attractive as peat alone to the broilers in the field study (paper **I**). Nevertheless, it is likely that spatial separation of options preferred for different activities will have a greater benefit for animal welfare than mixing them in the same location, because this should stimulate physical skill development (dexterity, flexibility, balance), facilitate learning about differences (speeding adaptation when moved to a novel environment such as the laying house), and develop skills in decision-making.

In humans, the provision of too many choices is associated with psychological choice overload (Chernev et al., 2012; Scheibehenne et al., 2010), which may lead to longer cognitive processing, inability to choose or regretting the decision made. Too few or too similar choices may also result in negative affective states (Deci, 1980; Deci & Ryan, 1985). Providing an adequate number of choices, which are different enough to avoid generalisation, is thus important when using choice provision to enhance animal welfare. Based on the results (papers **I** and **II**), the chickens appeared to have no difficulty navigating among 4 to 7 variants of the same resource type, but it is unclear how they would respond to more or fewer options. A moderate number of choices (approximately 4 options per resource type) allows the choices to differ sufficiently to complement one another rather than heavily overlapping in their use for different functions, meaning they should be preferred for different activities. Yet, experienced individuals might be able to cope with a larger number or more complex, cognitively challenging choices than individuals with little decision-making experience. Thus, it might be necessary to adjust the choices provided as the individuals develop. This fits within the concept of boosting positive arousal through moderate challenge to achieve a state of flow during bursts of activity interspersed with relaxation (Hintze & Yee, 2023; Meehan & Mench, 2007). It is as yet unclear how to balance relaxed states resulting from satisfaction of wants with short-term, highly active positive states where the pleasure component is high during interaction with liked resources.

5.6 Conclusions

In this thesis, I examined the effects of resource choice on the behaviour and other welfare-indicators of poultry (broiler chickens and laying hens). The thesis documents that resource choice, in the form of different types of litter with different characteristics, varied in their degree of attractiveness depending on the behavioural motivation of broiler chickens (paper **I**). Furthermore, resource choice, when provided as four different perch- and litter types during rearing, was associated with higher levels of positive-valence behaviours and lower levels of negative-valence behaviours in layer pullets. Some resource choice effects also extended into adulthood. The behavioural differences were associated with greater body weight and better plumage condition but without effects on comb quality (papers **II** and **III**).

Refreshment was found to have a large impact on the attractiveness of the litter choices provided in the field study (paper **I**), and the practise of refreshment of litter was likely important for maintaining the treatment differences throughout the experimental study when balanced against the stable presence of the different perch types used for resting and feeling secure (paper **II** and **III**).

Provision of resource choice during development had both short -and long lasting, positive effects on the behaviour and welfare of poultry. The present results may serve as a foundation for the practical implementation of resource choice in poultry production and inspire farmers to provide more complex environments to animals.

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7 Articles

I A buffet of litters – Broiler chickens behave differently according to litter type and freshness

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A buffet of litters – Broiler chickens behave differently according to litter type and freshness

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Exploratory behaviour

ABSTRACT

Poultry are usually provided with a single litter type that may not satisfy all litter-directed behavioural functions and may lose functional value over time due to soiling and degradation. We investigated this hypothesis in two commercial broiler flocks reared to 34 days of age on peat litter. We provided "litter buffets" comprising seven adjacent 1-m^2 litter trays, each containing a different litter type (peat, peat mixed with sawdust, wood shavings, finely-chopped bark, oat straw pellets, coarsely-crushed rape straw, and finely-ground rape straw), in two different locations of the house. We observed the number of chickens performing different behaviours, and total number present, in each litter tray once weekly for four weeks, before and after adding 5 L of fresh material. The litter types varied in attractiveness for ground scratching (an exploratory component of foraging behaviour; $P < 0.001$), with the most ground scratching occurring in peat, mixed peat, wood shavings, and fine straw (in order of preference). More ground scratching also occurred in fresh than used litter ($P < 0.001$). Dustbathing varied according to the interaction between litter type and freshness ($P = 0.029$), with mixed peat, peat, fine straw, and wood shavings being preferred when fresh, and fine straw best retaining dustbathing attractiveness when used. Lying resting varied between litter types ($P < 0.001$), being highest in wood shavings, mixed peat, and fine straw regardless of freshness and, because lying resting was the most common activity, the total chickens present followed the same pattern. The proportion of small particles ($\leq$1.0 mm diameter) increased to varying extents between litter types as litter degraded from fresh to used. Ground scratching was not associated with particle size distribution. However, chickens preferred to dustbathe in fresh litter with more small particles ($P < 0.001$), and in used litter with fewer large particles (>3.6 mm; $P = 0.023$). They also tended to avoid lying resting in fresh litter with a high proportion of large particles ($P < 0.001$). Consistent with our hypothesis, different litter materials stimulated different behavioural responses and top dressing with fresh litter increased litter attractiveness for ground scratching and dustbathing. We conclude that broiler welfare can be improved by providing a choice of litter materials to address different motivational priorities. Although chickens were attracted to peat and mixed peat for ground scratching and dustbathing, to avoid loss of peatlands (an environmental sustainability concern), wood shavings can be the main litter type, supplemented by patches of fine rape straw, with periodic top dressing of both.

1. Introduction

In poultry production, a single litter type is typically provided on the house floor. Litter is used to improve housing conditions by providing insulation, cushioning from the hard floor surface, manure dilution, and moisture absorption (Monira et al., 2003). Litter may also be provided as an environmental enrichment to stimulate activity and displays of natural behaviour in broiler production (Vas et al., 2020). Behaviourally, litter is an attractive resource for foraging, resting, and dustbathing (Petherick and Duncan, 1989; Toghyani et al., 2010), with some litter materials being more preferred for particular behaviours than others (Shields et al., 2005; Toghyani et al., 2010; Baxter et al., 2018a). Such preferences probably arise due to differences in the effectiveness and associated reward value of using different materials for the performance of a behaviour having a specific function. For example, van Liere (1992) provided evidence that a fine-grained, absorbent, lipophilic litter material is both attractive for dustbathing and effective for removing stale lipids from feathers through dustbathing. Thus, provision of only one

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litter type may not accommodate all the different functions for which litter is used by the birds. Moreover, providing a choice of litter materials would allow birds to choose their most preferred litter substrate for different behaviours, which may increase their perceived control over their environment resulting in better animal welfare (Wiepkema and Koolhaas, 1993; Leotti et al., 2010).

Litter preferences for the performance of specific behaviours may change as the litter breaks down and accumulates moisture and faecal matter. In support of this concept, it has been reported that laying hens preferred to dustbathe in used rather than fresh wood shavings (Moesta et al., 2008) but to forage in clean feed than in feed soiled with excreta (von Waldburg-Zeil et al., 2019). Further, broiler chickens performed more foraging in regularly replenished than unreplenished wood shavings (Baxter et al., 2018a) even though they did not discriminate between soiled and clean litter when required to work for access to it (Monckton et al., 2020). These findings suggest that providing one litter type, fresh only at the start of the production cycle, or recycling the same litter across successive flocks, may not satisfy all of the behavioural functions of litter. However, there is limited information regarding how the use of different litter types for different behaviours is affected by particle size and soiling, and whether some materials maintain their attractiveness for particular behaviours for longer than others.

Peat has been reported to be an attractive litter material for foraging and dustbathing (de Jong et al., 2007; Baxter et al., 2018a). In Scandinavia, wood shavings litter is the most commonly used litter material, but peat is also provided in many broiler houses, both as whole-house litter and as an environmental enrichment material in houses with wood shavings litter (BenSassi et al., 2019; Vas et al., 2020). Alas, extraction of peat raises concerns from the perspective of environmental sustainability and climate change (Nugent et al., 2019; Loisel et al., 2021), and there is a need to find alternatives that fulfil its value as a poultry litter material.

The aim of this study was to investigate the behavioural responses of broiler chickens to seven simultaneously presented litter types under two conditions: used and fresh. The seven litter materials were peat (control), peat diluted by mixing it with sawdust, plain wood shavings, finely-chopped pine bark, oat straw pellets, coarsely-crushed rape straw, and finely-ground rape straw. These materials were selected based on availability, differences in composition, expected suitability as chicken litter, and potential for replacing peat. We hypothesised that chickens would benefit from access to more than one litter type, and periodic refreshment of litter materials (top dressing), due to differences in litter composition and, thus, functional value for different litter-directed behaviours. We examined this hypothesis by quantifying three behaviours performed on the litter: ground scratching (an exploratory component of foraging behaviour), dustbathing, and lying resting, as well as overall numbers of chickens using the different litter materials. We predicted that the number of birds using the different litter types would vary depending on the behaviour being performed and the freshness condition of the litters. However, it was unclear how birds would trade off a predicted preference for the cleanliness of fresh litter with a predicted preference for more broken-down (used) litter having smaller particle sizes, raising the possibility of interactions between litter type and freshness depending on the behaviour.

2. Methods

2.1. Ethics

This study complied with the Norwegian Regulation on Animal Experimentation (Forsøksdyrforskriften, 2015) under the Norwegian Animal Welfare Act (Dyrevernloven, 2009). No actions in this study were expected to lead to harm to the animals. Animal care and use was in accordance with normal commercial practices in Norwegian broiler production.

2.2. Animals, housing, and management

We collected data from two consecutive flocks of mixed-sex Ross 308 broiler chickens in a single house (60 ×20 m) at a commercial farm in south-eastern Norway. Both flocks had around 17,000 chickens from the same hatchery (Flock 1: 17,021; Flock 2: 17,099), placed as day-old chicks and kept to 34 days of age (mean liveweight and mortality, Flock 1: 2127 g, 3.81%; Flock 2: 2107 g, 4.35%). The chickens were raised on coarse peat litter (varying in degrees of decay) over a concrete floor with underfloor heating. The house was cleaned and disinfected prior to each flock being introduced into the house. Starting with continuous light, the lighting was gradually adjusted to a schedule of 17 h light:7 h dark by one week of age, after which the lighting was supplemented by natural light through windows. Additional environmental enrichments, including boxes and hay bales, were provided after the birds were 3 weeks old. The flocks were reared according to standard production practices in Norway, with *ad libitum* feed and water.

2.3. Experimental design

We placed two sets of seven adjacent 1 m^2 litter trays ("litter buffets"; Fig. 1) at opposite ends of the house between water and feeder lines. The trays were created using 10 cm diameter polyvinyl chloride pipe frames laid on the bare floor and divided by 10 cm-high cardboard partitions. Within a buffet, each litter tray contained a different litter type (Table 1). The order of litter types varied randomly across buffets and flocks. We placed the trays adjacent to one another in a buffet to increase the likelihood of an individual sampling multiple litter types. The chickens had continuous access to the buffets from the first week of age, when they were supplied with fresh litter approximately 3 cm deep. Subsequently, we top dressed the trays with fresh litter material only during observations.

2.4. Data collection

We made direct observations once weekly for four weeks per flock when the birds were between 1and 5 weeks of age. Trays within buffets were observed in a pre-determined balanced order. The two buffets were observed simultaneously by two experienced observers for 31.5 min before, and 31.5 min after, addition of 5 L of fresh material to each tray. To avoid disturbing the birds, the observers stood approximately 2 m away from the buffets. The observers practised the methods prior to data collection to ensure inter-observer reliability and balanced their observations of each buffet across weeks. The behaviours selected for observation (Table 2) were distinctive and easy to detect and record during direct observations.

An observation of a litter tray started with an instantaneous scan of the total number of birds in the tray and the number lying. This was followed by a 15-s 1–0 scan to determine the numbers of ground scratching and dustbathing birds. Every 45 s, the observer slowly moved over and scanned the next tray on the list, continuing until 6 scans/litter type had been completed. The fresh litter was then poured into each tray (from pre-prepared containers placed nearby) and the sampling procedure was repeated. Prior to statistical analysis, we subtracted the

Fig. 1. Broiler chicks exploring a litter buffet offering different litter types (Photo: Ruth C. Newberry).

Table 1
Seven different litter types provided in each litter buffet.

Litter type	Contents	Reason selected	Product name (Company)
Peat	Fine-grained peat, *Sphagnum* moss species	Attractive to poultry, fine-grained, absorbent	Strøtorv Ren (Tjerbo Torvfabrikk AS, Rakkestad NO)
Mixed peat	70% fine-grained peat, 30% sawdust (spruce and pine)	Dilution of peat to reduce peat usage	Degernes strøtorv med 30% flis (Degernes Torvstrøfabrikk AS, Degernes, NO)
Wood shavings	Dried wood shavings (spruce and pine)	Commonly used poultry litter, insulative, cushioning, absorbent	Kutterspånbal (Staben, SE)
Bark	Dried sanitary pine bark, finely-cut	Fine-grained, absorbent	Plumbo Natur Fin Furubark (Krefting & Co, Vøyenenga NO)
Pellets	61% oat straw, 20% ground oat hulls, 12% limestone, 5% wheat bran, 2% fat, whole pellets	Commercial poultry litter product, durable, easily handled, absorbent	Strøbad (Felleskjøpet, Oslo NO)
Coarse straw	Coarsely-crushed, heat-treated, rape straw	Commercial poultry litter product, durable, easily handled, absorbent	AJ-Kross Grov (AJ Energi & Strø, Aulum DK)
Fine straw	Finely-ground, heat-treated, rape straw	Commercial poultry litter product, fine-grained, absorbent	AJ-Kross Fin (AJ Energi & Strø, Aulum DK)

Table 2
Ethogram.

Behaviour	Description	Measure
Ground scratching	While standing, bird performs unilateral kicking movements with a foot, raking the substrate backwards with toes and claws. Usually repeated with alternating legs.	Number of birds performing behaviour (1–0 occurrence) within a 15-s scan
Dustbathing	While lying, bird rapidly lifting wings up and down multiple times while holding them close to body and scooping loose litter up into feathers (vertical wing shaking). May be accompanied by raking movements of feet in litter while lying. Bird may also be seen lying on side of body with legs stretched out and feathers flat but containing dust (side lying).	Number of birds performing behaviour (1–0 occurrence) within a 15-s scan
Lying resting	Bird lies with body resting on litter inside a tray. Legs are not supporting weight. Legs can be underneath body or stretched out. Excludes dustbathing birds.	Number of birds lying in an instantaneous scan minus number dustbathing
Total birds	Number of birds with at least one foot in contact with litter in a tray.	Total number of birds present in an instantaneous scan

mean number of birds dustbathing from the number lying per scan to get the number lying resting (Table 2).

In week 5, we collected samples of used litter from surface to floor in the centre of each litter tray (approximately 250 g/litter type) for comparison of particle size distribution with corresponding fresh litter samples taken from the product container. We passed three 50-g samples of each litter material in each freshness condition through a stack of eight sieves with progressively smaller holes, ranging from 5.6 mm to 0.2 mm diameter, to determine the amount in each of the resulting nine fractions (to the nearest 0.1 g), and mean fraction weight across the nine fractions. We then collated the data into three particle size fractions: ≤ 1.0 mm (small particles), 1.1–3.6 mm (medium particles) and > 3.6 mm (large particles) to determine the proportion of small, medium and large particles in each sample.

2.5. *Statistical analysis*

2.5.1. *General*

We conducted statistical analyses in R 4.2.2 (R R Core Team, 2022) with statistical significance set at $P < 0.05$. Each response variable was analysed in a mixed model using the lme4 (Bates et al., 2015, 2014) and lmerTest (Kuznetsova et al., 2017) packages. Model fit was evaluated using AIC and residual diagnostic plots, computed using the DHARMa package (Hartig, 2022). Type II and Type III Wald Chi-square tests were conducted with the car package (Fox and Weisberg, 2019). We used the stat package (R R Core Team, 2022) to assess explanatory variable significance ($\alpha = 0.05$), after which we computed planned pairwise comparisons using the emmeans package (Lenth, 2022). Least squares means were back-transformed to the response scale for presentation.

2.5.2. *Behavioural data*

We evaluated scan data (number of birds ground scratching, dustbathing, and lying resting, and in total, summed over the six scans per litter tray within freshness condition) using Generalized Linear Mixed Models (GLMM) with maximum likelihood estimation using Laplace approximation. A negative binomial distribution was specified to address overdispersion. We evaluated the effects of litter type, freshness condition and their interaction, with buffet nested within flock as a random factor. To control for changes with age, the age in weeks was included in the model as a continuous variable. If the interaction term was non-significant, it was removed from the final model.

2.5.3. *Litter composition*

We evaluated the effects of litter type on mean particle size fraction weight using General Linear Models (GLM) with Gaussian distribution separately for the two freshness conditions. To assess how birds responded to litter varying in proportions of small and large particles within each freshness condition, we used GLMMs with maximum likelihood estimation with Laplace approximation. Buffet nested within flock was included as a random effect, and a negative binomial distribution was specified to address overdispersion.

3. Results

3.1. *Ground scratching*

There was no interaction between litter type and freshness condition on the number of ground scratching birds (χ^2_6 =3.62; P = 0.728). Peat and mixed peat had the highest numbers of ground scratching birds (back-transformed mean ± 95% CI: peat 1.77 [1.3, 2.4], mixed peat 1.62 [1.2, 2.6]), followed by wood shavings (1.07 [0.7, 1.6]) and fine straw (0.90 [0.6, 1.4]), with the fewest in bark (0.56 [0.3, 0.9]), pellets (0.40 [0.2, 0.7]), and coarse straw (0.65 [0.4, 1.0]; litter type: χ^2_6 =41.36, P < 0.001; Fig. 2a). More birds ground scratched in fresh (1.55 [1.3, 1.9]) than used litter (0.50 [0.4, 0.7]; χ^2_1 =47.16, P < 0.001; Fig. 2b). Ground scratching also declined with increasing age (z = −2.10, χ^2_1 =4.39, P = 0.036).

3.2. *Dustbathing*

The number of dustbathing birds was affected by the interaction between litter type and freshness condition (χ^2_6 =14.08, P = 0.029; Fig. 3). In used litter, more birds were observed dustbathing in fine straw

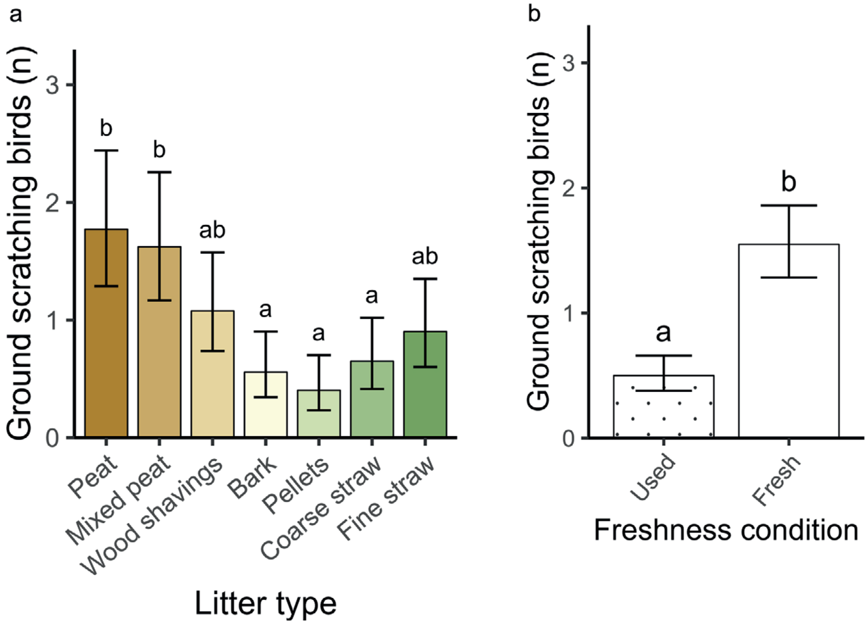

Fig. 2. Number of broiler chickens ground scratching (back-transformed mean ± 95% CI) in a) seven different litter types, and b) two freshness conditions. Data on chickens in two commercial flocks, observed weekly from 1 to 4 weeks of age in each of two litter buffets, were summed over 6 scans/litter type/freshness condition/week/buffet/flock. Letters indicate significant differences (P < 0.05).

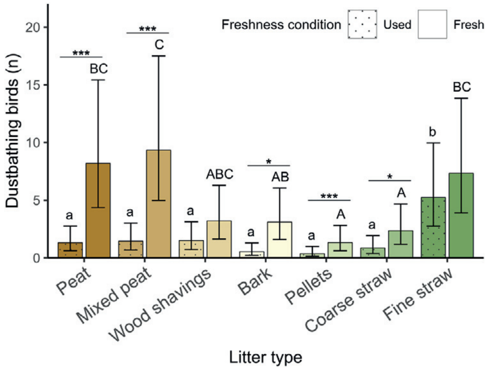

Fig. 3. Number of broiler chickens dustbathing (back-transformed mean ± 95% CI) in seven different litter types before (used litter) and after (fresh litter) adding 5 L of fresh litter. Data on chickens in two commercial flocks, observed weekly from 1 to 4 weeks of age in each of two litter buffets, were summed over 6 scans/litter type/freshness condition/week/buffet/flock. Lowercase letters indicate differences between litter types when used, and uppercase letters when fresh (P < 0.05). Stars indicate differences between used and fresh litter of the same type (*** P < 0.001, * P < 0.05).

(back-transformed mean ± 95% CI: 5.24 [2.8, 10.0]) than in all other litter types. When fresh, the highest numbers were observed dustbathing in mixed peat (9.34 [5.0, 17.5]), peat (8.21 [4.4, 15.4]), and fine straw (7.35 [3.9, 13.8]), with intermediate numbers in wood shavings (3.22 [1.6, 6.3]) and bark (3.12 [1.6, 6.1]), and the lowest numbers in pellets (1.34 [0.6, 2.8]) and coarse straw (2.36 [1.2, 4.7]). Fresh litter stimulated more dustbathing than used litter overall (used 1.16 [0.7, 1.9]; fresh 4.05 [2.6, 6.4]; $\chi^2_1 = 21.37$, $P < 0.001$), but not for all litter types. More birds were observed dustbathing in peat ($P < 0.001$), mixed peat ($P < 0.001$), bark ($P < 0.001$), pellets ($P = 0.022$), and coarse straw ($P = 0.025$) when fresh than used whereas birds did not clearly discriminate between fresh and used wood shavings ($P = 0.066$) or fine straw ($P = 0.337$) when dustbathing. Overall, fine straw (6.21 [3.6, 10.6]) was the most preferred, and pellets (0.71 [0.4, 1.4]) the least preferred, for dustbathing (litter type: $\chi^2_6 = 43.49$, $P < 0.001$). Age did not affect the number of birds observed dustbathing ($\chi^2_1 = 1.14$, $P = 0.285$).

3.3. *Lying resting*

The interaction between litter type and freshness condition did not affect the number of birds lying resting ($\chi^2_6 = 1.47$, $P = 0.961$) but there was a main effect of litter type ($\chi^2_6 = 62.19$, $P < 0.001$; Fig. 4a). The number lying resting in wood shavings (back-transformed mean with 95% CI: 72.6 [47.4; 111.0]) was higher than in all other litter types except mixed peat (56.1 [36.6, 85.9]) and fine straw (54.9 [35.8, 84.0]). Intermediate numbers were observed in peat (41.3 [26.9, 63.3]) and bark (43.1 [28.1, 66.0]). The fewest birds lay resting in pellets (22.8 [14.9, 35.1]), differing from the numbers in all other litters except coarse straw (33.4 [21.8, 51.2]). Neither the litter freshness condition ($\chi^2_1 = 0.0007$, $P = 0.978$) nor age ($\chi^2_1 = 0.0002$, $P = 0.989$) affected the number of birds lying resting.

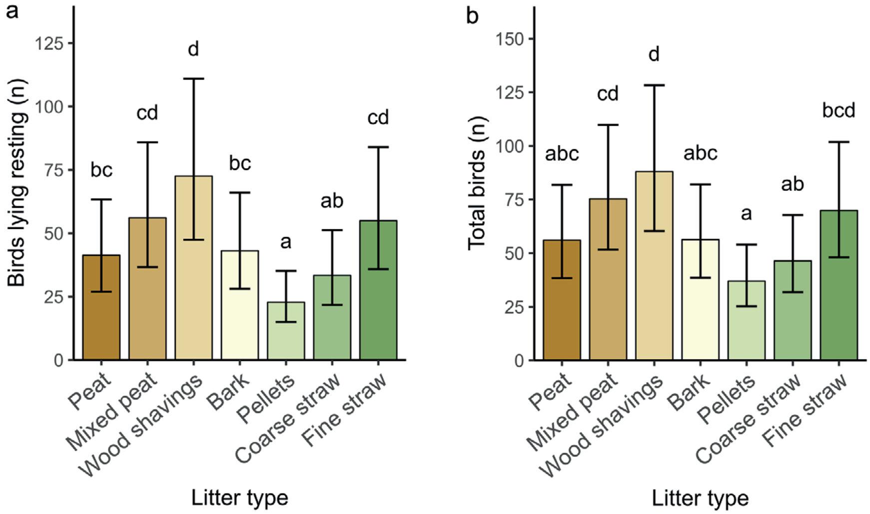

Fig. 4. Number of broiler chickens per litter type (back-transformed mean ± 95% CI) a) lying resting, and b) in total (data on chickens in two commercial flocks, observed weekly from 1 to 4 weeks of age in each of two litter buffets, summed over 6 scans/litter type/freshness condition/week/buffet/flock). Letters indicate significant differences ($P < 0.05$).

3.4. Total birds

The total number of birds present in the litter trays was unaffected by the interaction between litter type and freshness condition ($\chi^2_6 = 0.88$, $P = 0.989$) but did differ between litter types ($\chi^2_6 = 50.46$, $P < 0.001$; Fig. 4b), being highest in trays containing wood shavings (back-transformed mean ± 95% CI: 88.0 [60.3, 128.2]), followed by mixed peat (75.3 [51.6, 109.8]) and fine straw (69.9 [48.0, 101.9]). Intermediate numbers were observed in peat (56.0 [38.4, 81.8]) and bark (56.3 [38.6, 82.1]), and the lowest numbers in pellets (37.0 [25.3, 54.0]) and coarse straw (46.4 [31.8, 67.7]). Freshness condition ($\chi^2_1 = 3.80$, $P = 0.051$) and age ($\chi^2_1 = 0.03$, $P = 0.861$; maximum number of birds/tray in Weeks 2–5 was 46, 34, 52 and 36, respectively, across both flocks) had no effect on the total number of birds in the different litter materials.

3.5. Litter composition

Mean (± 95% CI) particle size fraction weight (g) was affected by

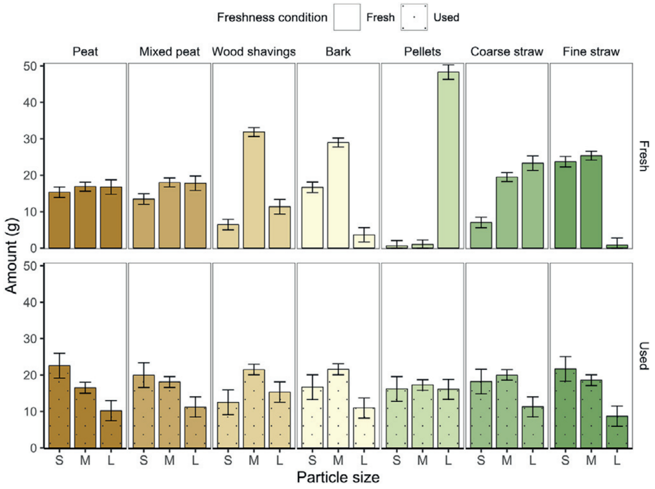

Fig. 5. Weight (g) of litter in each of three particle size fractions (S = ≤ 1.0 mm; M= 1.1–3.6 mm; L = > 3.6 mm) by litter type after four weeks of use by two commercial flocks of broiler chickens (least-square mean of three samples/flock ± 95% CI) and when fresh from the product container.

litter type in both fresh litter ($F_{6,\ 14}$ =132.39, $P < 0.001$) and used litter ($F_{6,\ 35}$ =4.36, $P = 0.002$). The materials varied in their proportion of small, medium, and large particles depending on freshness condition (Fig. 5). The number of ground scratching birds was not associated with the proportions of small or large particles in the litter, whether used (small: χ^2_1 =0.13, $P = 0.723$; large: χ^2_1 =0.38, $P = 0.537$) or fresh (small: χ^2_1 =1.84, $P = 0.175$; large: χ^2_1 =0.14, $P = 0.705$). However, birds preferred to dustbathe in used litter with a lower proportion of large particles ($\widehat{\beta} = -16.15$, $\chi^2_1 = 5.17$, $P = 0.023$) and in fresh litter with a higher proportion of small particles ($\widehat{\beta} = 4.36$, $\chi^2_1 = 14.01$, $P < 0.001$). They preferred to lie resting in used litter with a lower proportion of small particles ($\widehat{\beta} = -4.02$, $\chi^2_1 = 4.24$, $P = 0.039$), and in fresh litter with a lower proportion of large particles ($\widehat{\beta} = -1.49$, $\chi^2_1 = 16.66$, $P < 0.001$). When used, the total number of birds present in the litter materials was not significantly associated with either the proportions of small- (χ^2_1 =3.65, $P = 0.056$) or large (χ^2_1 =3.08, $P = 0.079$) particles in the litter but, when fresh, litter materials with a lower proportion of large particles attracted the most birds in total (χ^2_1 =9.95, $P = 0.002$).

4. Discussion

4.1. Overview

We investigated the behavioural responses of broiler chickens to seven different litter types presented simultaneously and evaluated how litter freshness condition (used or fresh) affected these responses. In brief, we found variation in the use of different litter types for different behaviours and a preference for fresh litter over used litter for active behaviours (ground scratching and dustbathing). We also found an association between litter particle size distribution and the performance of dustbathing and lying resting.

4.2. Ground scratching

Peat was the most attractive litter type for ground scratching whereas bark, pellets and coarse straw were the least preferred for this behaviour. However, the occurrence of ground scratching in peat, mixed peat, wood shavings, and fine straw did not differ significantly at the group level. The lack of a single overridingly preferred material for ground scratching is consistent with the interpretation that chickens are motivated to explore a variety of litter types rather than gravitating towards only a single type. Given that the four most preferred materials varied in particle size distribution, it is not surprising that we found no association between particle size distribution and the number of birds ground scratching. This result suggests that particle size *per se* is not an important factor influencing ground scratching.

Our results are not directly comparable with those of previous studies because litter preferences for ground scratching vary according to the specific choice of materials offered. For example, Villagrá et al. (2014) reported that broilers scratched most in rice hulls and least in sand, with wood shavings and straw intermediate, and Guinebretière et al. (2014) found that laying hens scratched more in wheat bran than peat, coarse construction sand or fine shell sand. Although ground scratching is accompanied by ground pecking when poultry are foraging, Guinebretière et al. (2014) reported a difference in litter preferences between these two behaviours, with both wheat bran and fine shell sand being preferred over coarse construction sand for ground pecking, with peat intermediate. Other studies lumping ground scratching with ground pecking, have reported a preference to forage in sand over wood shavings (laying hens, Shields et al., 2005), paper rolls, sand or wood shavings over rice hulls (broilers, Toghyani et al., 2010), or wood shavings, hemp shavings, and fine-grained sand over peat and pellets, with long-cut straw intermediate (laying hen chicks, Skånberg et al., 2021). However, adult laying hens worked a similar amount for access to different litter materials (peat moss, sand, wood shavings) in which they subsequently foraged (de Jong et al., 2007), suggesting that litter type preferences for foraging are not very robust. We excluded sand from our study due to its cost and potential to damage equipment.

In the current study, ground scratching was over three times more prevalent in fresh compared to used litter, emphasising the potency of repeated addition of fresh litter to stimulate this behaviour, as observed with peat by Vas et al. (2020). This finding was probably influenced by the cleanliness of fresh litter, given that laying hens preferred foraging in clean feed than feed contaminated with faeces (von Waldburg-Zeil et al., 2019). A preference for ground scratching in multiple types of fresh rather than used litter extends the finding of Baxter et al. (2018a) that broilers foraged more in regularly replenished rather than unreplenished wood shavings. However, Jacobs et al. (2021) did not detect a difference in levels of foraging in fresh and recycled wood shavings, indicating that other factors played a more dominant role on foraging (or perhaps just ground pecking) in that study.

We found that ground scratching declined with age, as also observed by Rayner et al. (2020). This was probably related to the typical decline in mobility of fast-growing broilers with advancing age (*e.g.* Silvera et al., 2017; Norring et al., 2019) but could also reflect declining interest in exploring increasingly familiar litter materials. Maintaining novelty by rotating between different litter materials when replenishing litter, and scattering high-value food items in the litter (Ipema et al., 2020; Wood et al., 2021), may aid in maintaining ground scratching activity over time, possibly contributing to improved leg condition and litter quality.

4.3. Dustbathing

We observed that mixed peat, peat, fine straw, and wood shavings were the most preferred materials for dustbathing when fresh, and fine straw was preferred over all other litter types when used. The birds also preferred to dustbathe in fresh materials with a relatively high proportion of small particles, and in used litter with predominantly small and medium rather than large particles. These results correspond with those of previous research in which chickens preferred to dustbathe in certain relatively fine-grained litter materials (*e.g.* laying hens: peat *versus* sand, sawdust, and wood shavings, Petherick and Duncan, 1989; broilers: sand *versus* rice hulls, paper, and wood shavings, Shields et al., 2004; sand *versus* wood shavings, Shields et al., 2005). Our finding of a preference to dustbathe in fresh wood shavings rather than finely-chopped bark was therefore unexpected. This may have occurred because the bark particles were harder and perceived as less comfortable, a testable hypothesis for future investigations. Although the wood shavings, bark, and fine straw tended to have a higher proportion of large particles when used than when fresh, clumping was minor and all litter types remained friable.

Freshness increased dustbathing in most of the litter types, including peat and mixed peat. More dustbathing may have also occurred in the used peat and mixed peat in the litter buffets if used peat had not been available throughout the house, given that broilers worked harder for the opportunity to dustbathe in peat than in sand or wood shavings when tested in a consumer demand paradigm (de Jong et al., 2007). Also, broilers dustbathed more in peat than in oat hulls, straw pellets, and wood shavings when the main litter type in the house was wood shavings (Baxter et al., 2018a). Despite the popularity of peat, we found that fine straw was the most preferred dustbathing material after peat and mixed peat when fresh, and functioned the best of all the litter types as a dustbathing substrate when used, suggesting that it could be a suitable alternative to peat for stimulating dustbathing. Contrary to the observation that caged laying hens preferred to dustbathe in used rather than fresh wood shavings (Moesta et al., 2008), the broilers in our study did not clearly discriminate between fresh and used wood shavings for dustbathing, possibly because they were also able to dustbathe in other more fine-grained materials rather than being confined to wood shavings alone. Access to a choice of suitable litter materials may have helped to maintain dustbathing with increasing age by accommodating potential changes in litter preferences over time (Sanotra et al., 1995; Nicol et al., 2001; Shields et al., 2005; Baxter et al., 2018b).

4.4. Lying resting and total birds

The numbers of birds lying resting were highest in wood shavings, mixed peat, and fine straw, and lowest in pellets. As lying resting consumes a considerable proportion of the broiler time budget (*e.g.* Bailie et al., 2013), it is not surprising that the total number of birds in the different litter types followed the same pattern. Similar to our results, broilers preferred to sit in wood shavings and sand instead of rice hulls or recycled paper rolls (Toghyani et al., 2010), and young broilers rested more in wood shavings and straw than in rice hulls or sand (Villagrá et al., 2014). Baxter et al. (2018a) also observed more sitting inactive in fresh and used wood shavings, but also in straw pellets, than in peat and oat hulls.

Birds in the present study displayed no preference for fresh litter over used litter when lying resting, suggesting that other factors than cleanliness affected their choice of resting substrates. This may explain why Monckton et al. (2020) found no difference in the motivation of broilers to access either clean or soiled litter. The birds in our study preferred to lie in fresh materials with relatively few large particles. This finding is consistent with their lower likelihood of resting in fresh pellets and coarse straw, the materials having the highest proportion of large particles, and which may have felt lumpy and uncomfortable. When selecting among used materials, the birds tended to rest in those having relatively more medium-sized than small particles, corresponding to their preference to rest in wood shavings, a soft, fluffy material with good insulative properties and a pale colour having a somewhat camouflaging effect for white-feathered chickens. Irrespective of litter type, the walls of the litter trays provided some vertical cover that may have attracted birds to the litter buffets as a perceived safe place for resting (Newberry and Shackleton, 1997). The total (and maximum) number of birds using the litter trays did not decline with age, suggesting that access to the different litter types was not a limiting factor as the birds grew larger.

4.5. Replacing peat

While we and others (*e.g.* de Jong et al., 2007; Baxter et al., 2018a; Vas et al., 2020) have found peat to be an attractive litter material for poultry, especially for foraging and dustbathing, environmental sustainability should be considered when selecting litter. Peat is a relatively clean, organic material with low pH and high absorbency, and may be beneficial for gut health when ingested (Trckova et al., 2005). However, as an especially carbon-rich terrestrial ecosystem, peatland is threatened by land development and peat extraction (Nugent et al., 2019; Loisel et al., 2021). Consequently, peatlands should be conserved, and extraction limitations would likely increase prices due to limited supply. Alternatives to peat are therefore needed for use in poultry production.

In many countries, wood shavings are the most commonly available litter material, and our results show that wood shavings are indeed preferred as a resting substrate. Given that resting is the most prominent activity of broilers, wood shavings are appropriate as the main litter type in the house but less suitable for stimulating ground scratching and dustbathing unless fresh. To encourage these activities, patches of peat are commonly provided as a source of environmental enrichment in Norwegian broiler houses (Vasdal et al., 2019; Vas et al., 2020). We found that this function of peat could be achieved using peat diluted with sawdust, thus reducing peat use (and cost). Furthermore, the best candidate for completely replacing peat was finely-ground rape straw. It is unclear whether other straw products would be similarly effective but according to the company offering this product (AJ Energi & Strø, 2022, https://ajenergi.dk/rapsstroeelsen), rape straw is more absorbent than wheat straw due to its higher cellulose content, and less dusty due to its longer, wider fibres. We also note that the rape straw was heat-treated, rendering it bacteria- and fungi-free, which is relevant from a biosecurity standpoint. Untreated rape straw, and other litter types, may react differently to parameters such as humidity that could result in flourishing of fungal and bacterial colonies, potentially impacting chicken health. Baxter et al. (2018a), (2018b) have identified oat hulls as another potential alternative to peat, and Vasdal et al. (2019) noted benefits from providing lucerne hay as a foraging enrichment. Our results also show the value of periodic addition of fresh litter materials for promoting ground scratching and dustbathing, although further research is needed to establish optimal rates of refreshment.

5. Conclusions

In support of our hypothesis, we found that litter materials differing in type, particle size distribution, and freshness were preferred to varying degrees for different behaviours. Therefore, we recommend enriching the broiler environment by providing additional litter types that complement the main litter in fulfilling different behavioural motivations. For example, our results support providing wood shavings as the main litter to accommodate resting behaviour, along with finely-ground rape straw as an additional substrate favoured for dustbathing and exploration. To further stimulate exploration and provide opportunities for the expression of individual differences, more options can be provided. For dustbathing, these materials should be fine-grained, with minimal clumping. As fresh litter was preferred over used material for active behaviours (ground scratching and dustbathing), we recommend regularly adding fresh materials to the environment to promote activity and better health for broilers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data and R code used to produce the statistics and figures presented in this article are available in Github: https://github.com/RVHolt/BuffetOfLitters.git (Holt et al., 2022).

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II Resource choice during juvenile development contributes to long-term welfare in laying hens

Resource choice during juvenile development contributes to long-term welfare in laying hens

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Abstract

We hypothesised that living in an environment offering multiple vs single variants of different resources is rewarding, with long-term organisational effects on behaviour. We investigated this hypothesis in laying hen pullets (*Gallus gallus domesticus*, n=364) reared in pens with one or four litter and perch types, respectively (n=8 pens/treatment). After 4 weeks (Period 1), half of the pens were switched to the opposite treatment (Period 2). From 16-27 weeks (Period 3), all groups were kept in similar adult laying pens. We assessed frequencies of behaviour associated with pleasurable vs aversive affective states. In support of our hypothesis, the opportunity to choose between multiple litter and perch types when young was associated with higher levels of positively-valenced play in

Period 1 and dustbathing in Periods 2 and 3, and lower levels of negatively-valenced severe feather pecking in Periods 1 and 2 and aggressive pecking in Period 3. More choices in Period 2 also led to better plumage condition in Periods 2 and 3, and higher growth in Period 3. We conclude that the opportunity to choose among different litter and perch types, vs having access to one type of each, had long-term beneficial effects on laying hen welfare.

Key words: animal welfare, behavioural development, environmental enrichment, environmental complexity, resource choice, affective states, positive welfare

1. Introduction

To improve the quality of life of captive and domestic animals, there is increasing interest not only to alleviate suffering but also to promote positive affective states [1–4]. Providing an environment with multiple variants of different resources to choose between could be an important source of environmental enrichment, promoting positive affective states for animals with an evolutionary history of natural selection in complex, heterogenous environments. In humans, the opportunities for decision-making are reported to lead to a greater perception of freedom and control [5,6]. Similarly, housing with opportunities to make choices may give animals an increased sense of control over their environment [7], along with reward from using the selected option [8]. In contrast, lack of choice is perceived negatively by humans, lowering motivation and sense of control [5,6].

In animals it may contribute to boredom [9], as well as redirection of behaviour to inappropriate targets (e.g.[10]).

While some choice may be positive, a large number of choices may be associated with psychological choice overload in humans [11,12]. Factors influencing perception of choice include the degree to which options can be categorised according to usefulness, ease of evaluating trade-offs between choices and degree of time pressure to make a decision between available options [12]. Deciding among choices also depends on the cost of each option relative to the individual's immediate [13,14] and future needs. Memory of post-choice reward from similar decisions made in the past is relevant to current choices [15], which can be influenced by the extent to which prior choices were perceived as final or reversible [16]. These findings suggest that providing a moderate, but not overwhelming, amount of choice in captive and domestic animal environments is likely to be beneficial for animal welfare.

Long-term learning and epigenetic processes channelling future behaviour are influenced by characteristics of the habitat to which individuals are exposed during early life when habitat preference is imprinted [17,18]. Exposure to a more heterogeneous habitat in early life may result in establishment of a broader habitat preference template. Developing familiarity with a broad constellation of environmental stimuli when young should aid adaptation to future environmental change because a greater proportion of the stimuli encountered following the change are likely to be familiar. Familiarity reduces fear, thereby speeding exploration of the novel components of the changed environment [15,19]. Thus, a diverse early environment should have beneficial effects on adaptability by providing a broad foundation for subsequent decision-making when exposed to unpredictable novelty. Furthermore, because behavioural plasticity is higher in juveniles than adults due to more rapid neurological development [20,21], learning to cope with the cognitive challenge imposed by choice in the environment should be more effective in juveniles than adults. In animal husbandry, providing a choice of several variants of different resources (“resource choice”) during rearing may be particularly important. For example, in domestic fowl (*Gallus gallus domesticus*) of strains selected for high egg production, it is standard commercial practice to rear pullets (young females) in one environment and later move them to a different environment for the adult, egg production phase of their lives.

Assessment of indicators of positive and negative affective states would provide insights into the extent to which pullet welfare is affected by environmental heterogeneity. While affective states are subjective, coloured by how individuals perceive their environment and experiences, they can be inferred from the performance of the different behaviours that they motivate. A positively-valenced state of playfulness is inferred from play behaviour [22,23], which is more likely to occur under safe, resource-abundant, fitness-promoting environmental conditions [24,25]. Play involves having fun and has been hypothesised to serve an adaptive function by generating opportunities for learning to cope with future unexpected situations [25]. Play is most common in young individuals that have the most to learn [26], including chickens [27], and has been correlated with growth rate [28] and survival rate [29,30] in some species. Dustbathing is another positively-valenced behaviour of chickens [31], contributing to comfort and fitness by cleaning and fluffing up the feathers [32,33]. While play and dustbathing are suppressed under potentially unsafe environmental conditions, they rebound when conditions improve [27,34] and can, thus, serve as indicators of a current, acute state of positive welfare. If the prevalence of these behaviours is repeatedly found to be higher in one environment than another, based on spontaneous behaviour under undisturbed conditions and not just in response to an immediate change in conditions, higher levels can be interpreted to indicate a higher level of positive welfare in that environment at the flock level.

Forms of behaviour associated with negatively-valenced affective states in chickens include vigilance, aggressive pecking and severe feather pecking. Vigilance refers to alert behaviour expressed in response to potential danger (e.g. [35,36] and serves as an indicator of anxiety [37]. Aggressive pecking is associated with frustration of access to resources and escalated resource defence [38,39] while feather damage due to severe feather pecking impairs the thermoregulatory and flight ability of laying hens and can lead to cannibalism-related mortality [40,41]. Aggressive pecking and severe feather pecking are painful for the receiver if resulting in wounds or feather removal [42]. Thus, an elevated prevalence of these fitness-threatening behaviours within a flock serves as an indicator of negative emotional valence at the flock level.

The aim of this study was to examine long-term organisational effects of early access to a heterogeneous environment offering multiple choices of litter and perch types (“resource choice”) on the behaviour of laying hens. We chose laying hens as our model species because they are readily available and their rearing conditions are reported to have long-term effects on their behaviour and welfare (e.g. [43–47]). Over three age periods, we compared the behaviour of experimental groups kept in environments with one or four litter and perch types, respectively. We hypothesised that, if different resource variants vary in their utility for different functions, provision of multiple variants would promote reward across diverse activities, with long-lasting organisational effects on behaviour. Specifically, we predicted that access to multiple litter and perch types vs only a single variant of each resource would increase the frequencies of play and dustbathing behaviour (associated with positively-valenced affective states) and decrease the frequencies of vigilance, aggressive pecking and severe feather pecking behaviour (associated with negatively-valenced affective states). We also expected that a heterogenous environment offering multiple choices of litter and perch types (resource choice) would result in fitness-promoting outcomes including higher growth rates, less feather damage, fewer comb wounds, and lower mortality than found in a homogeneous environment with a single type of each resource. Further, we predicted that the effects of early environmental heterogeneity on behavioural frequencies and fitness-related outcomes would be long-lasting, persisting into adulthood when all birds were kept under similar conditions.

2. Methods

2.1 Animal, housing and management

The study was conducted at the Swedish Livestock Research Centre of the Swedish University of Agricultural Sciences in Uppsala, SE. All procedures were approved by the Uppsala Animal Experiment Ethics Board Number 5.8.18-11549/2017 and all authors complied with the ARRIVE guidelines. Bovans Robust chicks (n=364) were obtained from a local commercial hatchery. The chicks were pseudo-randomly assigned to one of 16 groups in a manner that resulted in similar average bird weight and standard deviation across groups. Four groups comprised 22 chickens and the remaining groups comprised 23 chickens. The groups were reared in separate pens from 1 day of age to 27 weeks of age, with twice daily routine care (see Supplementary Table S1 for products used). The study time span was divided into three age periods.

2.1.1 Period 1 (Week 1-4, chick rearing period)

The rearing pens (245 x 120 x 180 cm), all located in one room, were set up with a drinker line with a wire on top to discourage perching, two feeders placed on the floor, a hanging infrared heat lamp for localised brooding warmth, and four perches (each perch 120 cm in length) with initial height of 15 cm. Heavy brown paper covered the pen walls up to a height sufficient to block visual contact between pens. Litter (approximately 3 cm deep) was provided in four shallow plastic trays (71 x 35 x 3.5 cm), and on the concrete floor to avoid chilling of the chicks. The litter trays were cleaned and refilled at 1- to 7-day intervals as needed to maintain continuous access to litter within. At 3 weeks, the perches were raised to 45 cm, the water line was raised and the ground feeders were replaced with a round hanging feed hopper.

Starter feed and water were provided *ad libitum*. Room temperature, light schedule and ventilation were automatically controlled. Room temperature was kept at 25 °C. Lights were on for 20 h on Day 1, 18 h on Day 2, 16 h for the rest of the week, 14 h in Week 2, 13 h in Week 3 and 12 h in Week 4. Apart from a 15-min dawn and dusk at the start and end of each photoperiod, respectively, the mean light intensity in the pens was 18 lux at chick level (range: 7-37 lux).

2.1.2 Period 2 (Week 5-15, juvenile pullet rearing period)

At the beginning of Week 5, the heat lamp was removed, the perches were raised to 55 cm, the four shallow litter trays were exchanged for deeper trays (78 x 56 x 18 cm; 55 L) and litter was removed from the floor. The litter trays were cleaned and refilled once weekly to a depth of approximately 5 cm. The starter feed was exchanged for growing feed at 7 weeks of age. Room temperature was reduced to 20 °C at 8 weeks of age. The photoperiod was decreased by 1 h/week from 12 h to 10 h and then held stable.

2.1.3 Period 3 (Week 16-27, adult laying period)

In Week 16, all groups were transferred to new pens (362 x 356 x 297 cm) in another experimental room to simulate moving to a laying house. Each pen had two hanging feed hoppers containing layer feed, one bell drinker and two metal colony nests (115 x 46 x 30 cm each) accessible from a raised, sloping (-3.7°) plastic slatted floor area (230 x 356 x (maximum) 268 cm). At the back of the pen was a concrete-floored litter area (132 x 356 x 297 cm) containing crushed straw pellets as litter (approximately 5 cm deep). Three shallow plastic trays were attached to the wall as platforms (each 71 cm long; 70, 155 and 220 cm above the concrete floor). The pens also contained a wooden perch (120 cm long; 187 cm over slatted floor), a slanted perch stand with four plastic perches (each 155 cm long; set at 43, 96, 149 and 205 cm high, respectively, over slatted floor) and five low plastic perches (320 cm long, 3 cm high) attached to the slatted floor. Room temperature was kept at 20 °C and the mean light intensity was 5.4 lux at hen level (range: 3-8 lux). The photoperiod, including a 15-min dawn and dusk, was 10 h to 20 weeks, then increased by 1 h weekly to 14 h. It was then kept steady to the end of the experiment (27 weeks), when the hens were adopted out to local farmers and chicken enthusiasts.

2.2 Experimental design

In Period 1 (P1), pens were assigned to one of two environmental treatments (Fig. 1). Single-choice pens (S: n=8) had four perches of one perch type and four litter trays containing one litter type (with the same litter type on the floor). One perch type was paired with one litter type, resulting in four combinations that were balanced across pens (Fig. 2a). In this treatment, we were focussed on the general effects of having only one perch and litter type, regardless of differences in specific litter and perch characteristics. Multi-choice pens (M: n=8) had four different perch types and four different litter types (one type/tray; Fig. 2b). The relative location of each perch and litter type within the pen was balanced across M pens. The perch types were a pine plank (120x9.5x2 cm), a thick rope (three braided horse leads, 120x4x3 cm), a round rubber perch (120 cm long, diameter

3.5 cm) and a flat wire mesh perch in a spruce frame (120x13.5x1 cm, mesh openings 1 cm^2). The litter types were fine-grained sand (maximum 0.03 mm diameter), wood shavings (dust-extracted mixed wood, mostly spruce), straw (100 % wheat, long-cut) and peat (100 % Sphagnum moss). One of these litter types also covered the pen floor (balanced across pens). See Supplementary Table S2 for pen details.

At the beginning of Period 2 (P2), four pens/treatment were switched to the opposite treatment, resulting in four treatment combinations (Fig.1): S in both periods, S to M, M to S and M in both periods, arranged in a balanced block design with four blocks. After the move to the laying room (Period 3; P3), all groups were housed in similar pens (Fig. 2d) distributed across four blocks in a balanced block design. Blocking was done to control for possible small differences in environment across locations within experimental rooms.

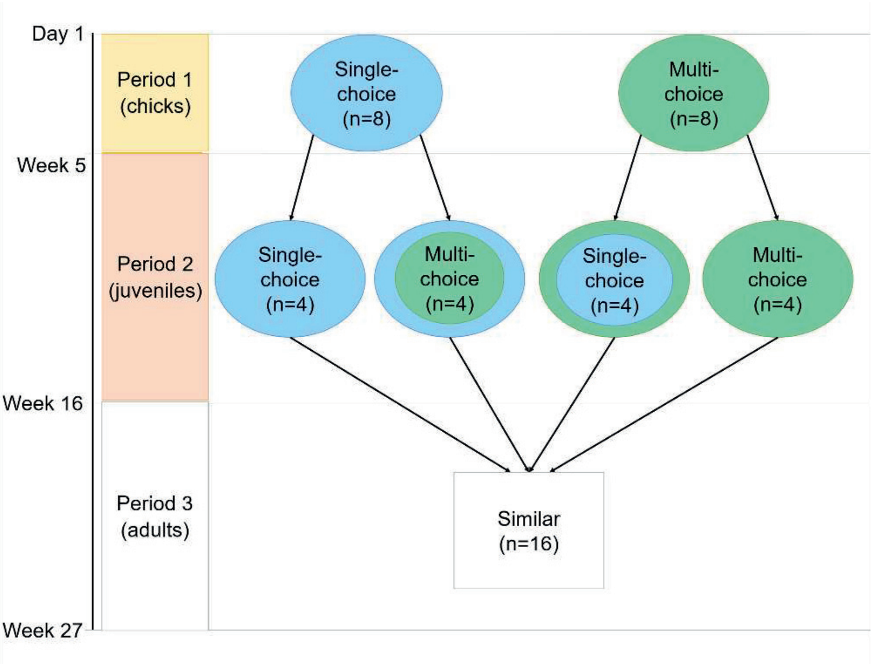
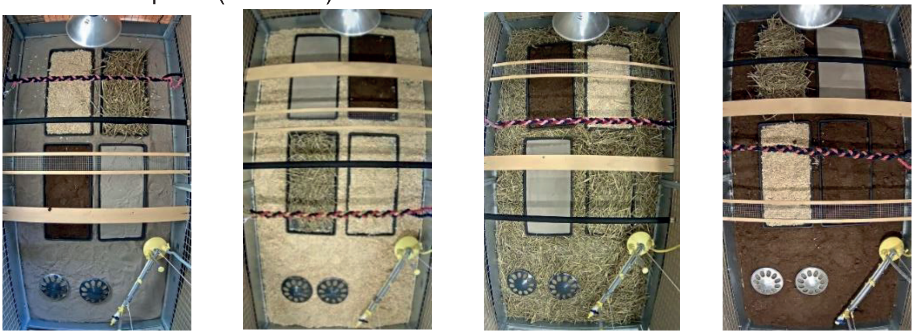


Figure 1. Experimental design. In Period 1, groups (n=16) were assigned to one of two treatments: Single-choice with a single perch and litter type or Multi-choice with four perch and litter types. In Period 2, half the groups were switched to the opposite treatment. All groups were moved to similar pens for Period 3.

a Single-choice pens (Period 1)

Plank + Sand | Rope + Shavings | Rubber + Straw | Wire + Peat

b Multi-choice pens (Period 1)



c Single-choice (Period 2) | Multi-choice (Period 2)

d Adult pen (Period 3)

Figure 2. Examples of treatments. **a)** Single-choice pens in Period 1 (Week 1-4), illustrating pens with different single perch- and litter types. **b)** Multi-choice pens in Period 1 with four perch and litter types. Type of floor litter and the location of perch and litter types were balanced across Multi-choice pens. **c**) A Single-choice and a Multi-choice pen in Period 2 (Week 5-15). Perch and litter type combinations as in Period 1 (litter in trays only). **d**) An adult laying hen pen in Period 3 (Week 16-27; all pens were similar).

2.3 Data collection

Direct observations of each pen were made by three experienced observers on one to three days each week from Week 3-23, with rounds of observations made across different times of day between 0900 and 1800 h, in a balanced order across pens. For each pen observation, the observer sat still in the aisle by the pen door for a 15-s familiarization period, then conducted an instantaneous scan of the number of vigilant pullets followed by a 3-min scan for the occurrence or number of events of the remaining behaviours in the ethogram (Table 1; Supplementary Table S3). Each pen was observed 23 times during P1 (n=368; 69 minutes/pen), 67 times in P2 (n=1072; 201 minutes/pen) and 18 times in P3 (n=288; 54 minutes/pen). Inter-observer concordance was monitored regularly and maintained at 90% or higher. The visual difference between treatments (Figure 2) prevented blinding to the treatment under observation.

Daily mortality was registered, and all birds were weighed in P1 (Day 17), and at the end of P2 (Day 108-109) and 3 (Day 184-186). At the time each bird was weighed in P2 and P3, damage to the feathers of two body parts (wings, tail) was scored and summed for statistical analysis. Comb wounds were also scored during the final weighing in Week 27 (Table 1).

Table 1. Response variables. Behavioural data were collected per pen. Health measures were collected per individual.

Response variable	Description	Variable as analysed
Behaviour		
Play	One or more birds performs sudden running and/or sparring. Appear unserious and non-harmful, without resulting in vocal “complaint” or persistent unilateral withdrawal.	Occurrence (1/0) within pen during 3-min scan.
Dustbathing	One or more birds performs vertical wing shaking, side lying and/or rubbing of the head in the litter while lying.	Occurrence (1/0) within pen during 3-min scan.
Vigilance	Bird sits or stands stationary on perch or floor, alert, with neck stretched and with either a fixed or rapidly moving gaze.	Proportion of birds vigilant within pen/instantaneous scan before each 3-min scan.
Aggressive pecking	One or more pecks at the head (comb to base of neck) of a conspecific with a stabbing or pulling movement, resulting in an immediate reaction by the receiver (moving away and/or vocalising sharply).	Number of pecks within pen/3-min scan.
Severe feather pecking	One or more pecks at the feathers or skin of a conspecific, at any body region excluding the head (comb to base of neck), with stabbing or pulling movement, resulting in an immediate reaction by the receiver (moving away and/or vocalising)	Number of pecks within pen/3-min scan.
Health		
Mortality	Daily deaths (found dead and culled) registered by pen.	Too rare for analysis.
Body weight	Each bird weighed to nearest g on Day 17 (Period 1), 108-109 (end of Period 2) and 184-186 (end of Period 3).	Body weight/bird/period (g).
Feather damage score	Proportion of split, frizzy and broken feathers, scored as 0 (<50 %) or 1 (≥50-100 %) for each of two body regions/bird (wings, tail), on Day 108-109 (end of Period 2) and 184-186 (end of Period 3).	Summed score/bird.
Comb wound score	Number of wounds on each bird’s comb, scored as 1 (0-3), 2 (4-6) or 3 (>6), on Day 184-186 (end of Period 3).	Score/bird.

2.4 Statistical analysis

All statistical analyses were completed in R 4.0.2 [48], with statistical significance set at $P<0.05$. Due to repeated measures within pens, all variables were analysed using mixed models with pen as a random effect. Residual diagnostics plots for mixed models, computed using the DHARMa [49] package, were used to evaluate and confirm model fit. Binary and count data were analysed with generalised linear mixed models (GLMM), whereby parameters were estimated based on maximum likelihood with Laplace approximation. A binomial distribution was applied to the 1/0 variables (playing, dustbathing). Overdispersion of the count variables (aggressive pecking, severe feather pecking) was addressed by specifying a negative binomial distribution. As the proportion of vigilant birds (vigilance) and cumulative body weight were continuous variables with Gaussian distribution, linear mixed models (LMM) were fit with restricted maximum likelihood (REML) and T-tests were conducted using the Satterthwaite approximation for degrees of freedom. The packages lme4 [50] and lmerTest [51] were used for all GLMM and LMM analyses and the emmeans package [52] was used for computing 95 % confidence intervals (CI). For each behavioural response in P1, we evaluated the effects of block, observer and P1 treatment and, in P2 and P3, we included block, observer, P1 treatment, P2 treatment and their interaction. Similar models were run for body weight but without observer. Models were re-run excluding block and the interaction term as these factors were non-significant. Observer was also removed from the behaviour models as no consistent trends were observed. A separate model was used to assess changes in each response variable across P1, P2 and P3. For presentation, proportions were transformed into percentages post-analysis (playing, dustbathing and vigilance).

Mortality was too low for statistical analysis. As feather damage and comb wound scores were ordinal variables with repeated sampling within pen, we used cumulative link mixed models (CLMM; ordinal package [53] fit with Laplace approximation to evaluate the effects of block, P1 treatment, P2 treatment and their interaction on feather damage scores in P2 and P3, and comb wounds in P3. Block, and the interaction term, were non-significant and so dropped.

Type II Wald Chi-square tests (GLMM - car package [54]; LMM – stat package [48]; CLMM – RVAideMemoire package [55]) were used to assess the significance of main effects for all models. Planned pairwise comparisons of significant fixed effects were performed using the emmeans package [52] before back-transformation of means to the response scale for presentation. For CLMM, this back-transformation required the mode “mean.class”. Mean.class transformed the ordinal levels of the response variable to a scale of 1-3 (due to three levels in ordinal response variables). Consequently, a constant of one (1.0) was subtracted from the pairwise comparison results of feather damage scores to transform the output to a scale of 0-2, a similar scale to the original summed scores. As comb wound scores already existed on a scale of 1-3, no post-pairwise comparison transformation was needed. Supplementary Table S4 provides an overview of models.

3. Results

Play was affected by treatment in P1 (X^2_1=0.52, P=0.018), occurring more often in the M treatment than in the S treatment (Fig. 3a). Neither the P1 treatment nor the P2 treatment affected the occurrence of play during P2 or P3 (P>0.05). The occurrence of play declined over periods (χ^2_2=124.67, P<0.001).

Dustbathing was observed more often in S than M pens during P1 (χ^2_1=7.90, P=0.005; Fig. 3b). P1 treatment did not affect dustbathing in P2 or P3 (P>0.05). However, the P2 treatment affected dustbathing in P2 (χ^2_1=6.80, P=0.009) and P3 (χ^2_1=4.70, P=0.030), with dustbathing occurring more often in the M than S treatment. Period also affected dustbathing (χ^2_2=11.13, P=0.004). There was no difference between P1 and P2, but dustbathing occurred more often in P3 than in P1 or P2.

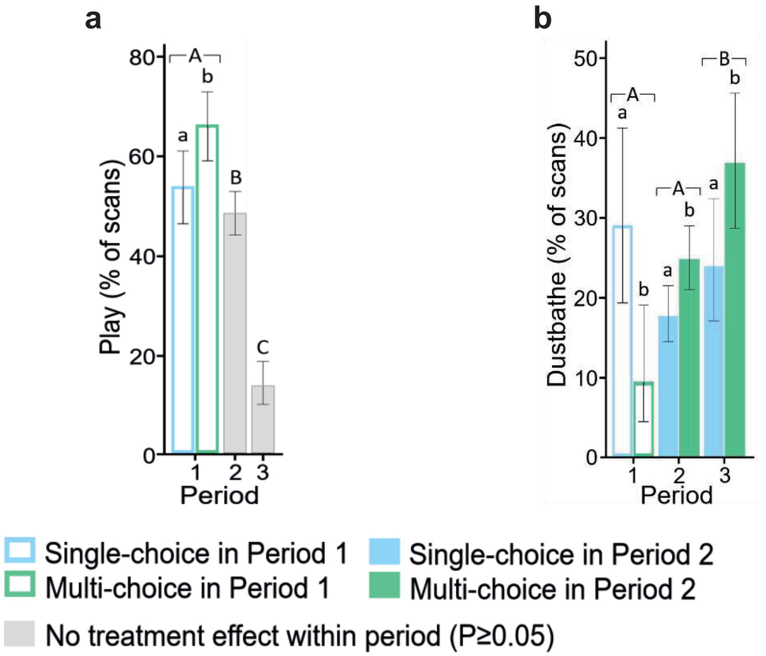


Figure 3. Behaviour (back-transformed mean % of scans with 95% CI) was associated with positive affective states across periods (1: Week 1-4; 2: Week 5-15; 3: Week 16-27): a) play and b) dustbathing. Bars with coloured outlines indicate an effect of the treatment experienced in Period 1 (Single-choice, blue; Multi-choice, green). Colour-filled bars indicate an effect of the treatment experienced in Period 2 (Single-choice, blue; Multi-choice, green). Uppercase letters indicate period differences and lowercase letters indicate treatment differences ($P<0.05$).

The proportion of vigilant birds/scan was not affected by current or previous treatment in any period ($P>0.05$) but did differ between periods ($F_{2,\ 1694}=189.66$, $P<0.001$; Fig. 4a). The highest level of vigilance occurred in P2, followed by P1 and P3.

The frequency of aggressive pecking/scan was not affected by treatment in P1 or P2 ($P>0.05$). However, the P1 treatment affected the frequency of aggression in P3 ($\chi^2_1=4.98$, $P=0.026$; Fig. 4b), with pullets reared in the M treatment performing less aggressive pecking than pullets reared in the S treatment. The P2 treatment did not affect the expression of aggressive pecking in P3. Period affected aggressive pecking ($\chi^2_2=50.26$, $P<0.001$), which increased as the pullets grew older.

The frequency of severe feather pecking/scan was affected by the P1 treatment ($\chi^2_1=12.93$, $P<0.001$; Fig. 4c), being lower in the M than in the S treatment. The P1 treatment did not affect severe feather pecking during P2 ($P>0.05$) but the P2 treatment affected severe feather pecking during P2 ($\chi^2_1=7.09$, $P=0.008$), whereby pullets kept in the M treatment exhibited a lower frequency of severe feather pecking compared to those kept in the S treatment. The frequency of severe feather pecking expressed in P3 was not affected by the treatment experienced in P1 or P2 ($P>0.05$). Period had an effect ($\chi^2_2=43.36$, $P=<0.001$), whereby pullets performed more severe feather pecking in P2 and P3 than in P1.

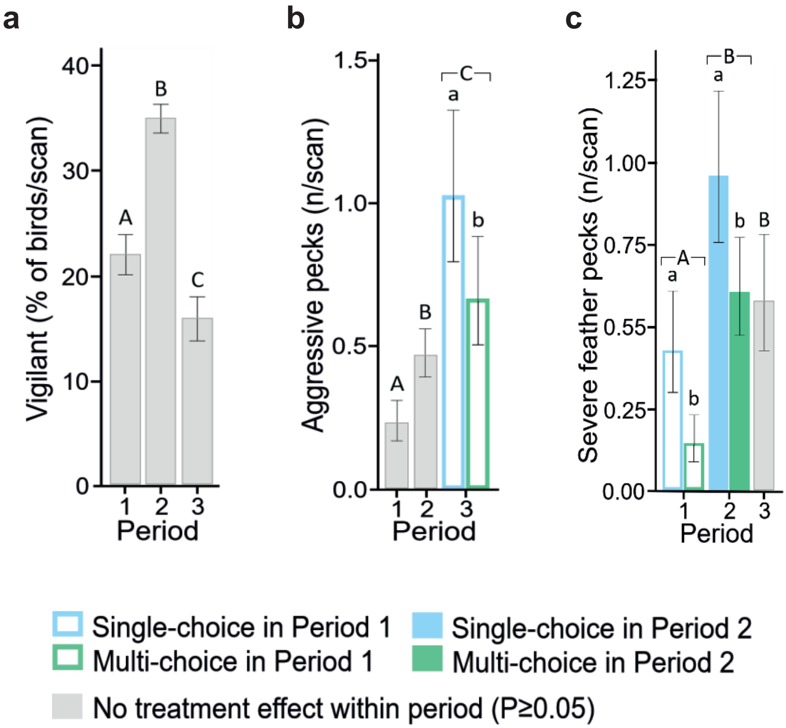


Figure 4. Behaviour associated with negative affective states across periods (1: Week 1-4; 2: Week 5-15; 3: Week 16-27): **a**) vigilance (back-transformed least squares mean % of birds/scan), **b**) aggressive pecking (back-transformed mean frequency/scan) and **c**) severe feather pecking (back-transformed mean frequency/scan), with 95 % CI. Bars with coloured outlines indicate an effect of treatment experienced in Period 1 (Single-choice, blue; Multi-choice, green). Colour-filled bars indicate an effect of treatment experienced in Period 2 (Single-choice, blue; Multi-choice, green). Uppercase letters indicate period differences and lowercase letters indicate treatment differences (P<0.05).

From Week 1-27, five pullets died or were removed from the experiment (1.37 % total mortality; P1: 0.55 %; P2: 0.83 %; P3: 0 %), with mortality unrelated to treatment (too low for statistical comparison). Body weights in P1 (Day 17) and P2 (Day 108-109) were not affected by treatment (P>0.05; Fig. 5a). However, pullets kept in the M treatment in P2 had higher body weights in P3 (Day 184-186) than those kept in the S treatment ($F_{1,\ 12.9}$=7.21, P=0.019).

Feather damage scores were not affected by the P1 treatment in P2 or P3 (P>0.05). Nevertheless, pullets kept in the M treatment in P2 had lower feather damage scores in both P2 and P3 (Fig. 5b) than pullets kept in the S treatment (P2: χ^2_1=8.77, P=0.003; P3: χ^2_1=9.64, P=0.002). Comb wounds in P3 were unaffected by treatment in P1 or P2 (P>0.05; arithmetic mean score with 95 % CI on a scale from 1 to 3: 2.22 [2.14, 2.29]).

See Supplementary Table S5 for back-transformed means with 95 % CI for each model, Supplementary Table S6 for odds ratios from significant pairwise comparisons, and Supplementary Table S7 for back-transformed mean with 95 % CI for each treatment combination.

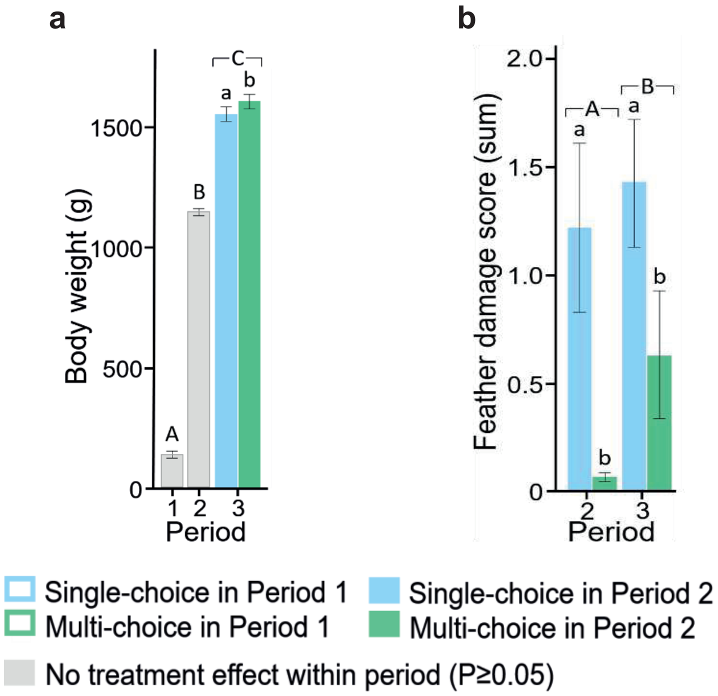


Figure 5. Health measures across periods (1: Week 1-4; 2: Week 5-15; 3: Week 16-27): **a**) cumulative body weight (back-transformed least squares mean g) and **b**) feather damage score (back-transformed mean of summed score), with 95 % CI. A score of 0 = <50 % of wing and tail feathers damaged, 1 = either wing or tail feathers ≥50-100 % damaged and 2 = both wing and tail feathers ≥50-100 % damaged. Colour-filled bars indicate an effect of treatment experienced in Period 2 (Single-choice, blue; Multi-choice, green). Uppercase letters indicate period differences and lowercase letters indicate treatment differences (P<0.05).

4. Discussion

We hypothesised that early life exposure to a more heterogenous environment with multiple litter and perch variants (resource choice) would be rewarding, promoting positive indicators of welfare, reducing negative indicators of welfare and having long-lasting organisational effects on behaviour. In support of this hypothesis, we found that pullets with the opportunity to choose between multiple litter and perch types displayed higher levels of positively-valenced behaviour (play and dustbathing), and lower levels of negatively-valenced behaviour (aggression and severe feather pecking), either at the time when multiple choices were offered or in adulthood when all hens were housed in a similar environment. These results were accompanied by indicators of higher fitness related to rearing in the M treatment (growth, feather condition), as expected if positive affective states reward and motivate fitness-promoting behaviour.

As predicted, pullets were more likely to play in M than S pens, though only in P1. The higher heterogeneity of the M treatment presumably provided more learning opportunities compared to the less diverse S treatment in P1, stimulating more play and higher levels of reward [25,56]. The lack of detected treatment differences in P2 and P3 may have been related to declining levels of spontaneous play with increasing age that are consistent with previous observations on chickens [27,57–59] and other species [26].

As predicted, the M treatment resulted in more dustbathing in P2, with carry-over into P3, though less dustbathing was recorded in the M than S treatment in P1. The M birds may have been more adept at selecting to dustbathe in more optimal litter materials in P2 and P3 following experience of dustbathing in the different litter choices in P1, when dustbathing bouts in less rewarding materials may have been truncated [33]. Overall, the number of scans in which dustbathing occurred was similar in P1 and P2, consistent with the observations of Keeling et al. [60] for the period 3-18 weeks. Dustbathing increased in P3 (adulthood), possibly related to the increased boldness of adult hens in lay [61,62] or increased plumage density and feather wear, thereby requiring more dustbathing to keep the plumage in good condition.

We expected that lack of resource choices would lower perceived control over the environment, resulting in higher anxiety as expressed by greater vigilance in S than M pens. However, vigilance results were not affected by choice provision in any period. This finding could be related to the fact that observations were conducted on undisturbed birds. It does not discount the possibility that choice provision would affect vigilance when exposed to a stressor. We did find that vigilance varied with age, being highest in P2. We propose that the young chicks were more focused on exploring their environment in P1, when they had the most to learn [25], and may have been less attentive for potential threats than when they were older. An increase in vigilance from P1 to P2 is consistent with a reported increase in pullet vigilance while perching between 3 and 15 weeks of age [35], possibly associated with moulting of juvenile plumage. The drop in vigilance from P2 to P3 may have been influenced by an increase in boldness in adulthood [61,62]. The larger pens in P3 also provided more space for avoidance of perceived threats.

We detected no differences in the frequency of aggressive pecks between treatments until P3 when, consistent with our prediction, pullets that had experienced the S treatment in P1 performed more aggressive pecks. Our finding of rising levels of aggressive pecking with age was probably related to the rise in reproductive hormones with approaching puberty as described by McKeegan and Savory [63], who reported an increase in the frequency of aggressive pecking in laying hens after the onset of lay. This increase in aggressive pecking likely explains why we only detected a treatment difference in P3. The treatment difference may have been influenced by the higher play experience gained by M pullets in P1, which could have promoted the development of improved social skills and more positive relationships among flock mates. The higher growth rate of M vs S hens in Period 3 is consistent with less aggression during this period. We did not detect a corresponding treatment difference in comb wound scores in P3, probably because the aggressive pecks varied in whether they inflicted skin damage or not. In small groups such as observed in the current study, a dominance hierarchy would be expected to emerge in P2 [64], following which a bird’s position in the hierarchy would likely have had the greatest impact on severity of comb damage.

Severe feather pecking was less frequent in the M than S treatment in P1 and P2 as predicted, although without carry-over to P3. The diverse litter types in the M treatment likely attracted and maintained interest in foraging in the litter whereas the single litter type in S pens may have been less effective in maintaining interest, leading to a shift in attention towards conspecific feathers, especially during juvenile moulting and the emergence of flight feathers. In P3, higher adult steroid levels may have overridden previous treatment effects on severe feather pecking, consistent with the observation that performance of this behaviour prior to the onset of lay is not a strong predictor of which hens will perform this behaviour in adulthood [65]. The higher levels of severe feather pecking in the S than M treatment in P2 were accompanied by higher feather damage scores in P2 that persisted in P3, presumably because there was no moulting and feather renewal during P3.

We detected no interactions between treatment in P1 and treatment in P2 overall. Depending on the variable, access to resource choice was beneficial in one period or the other and when present in both periods, the effects were neither magnified nor antagonistic. Further, across the measured variables, we found no support for a consistently stronger long-term effect of treatment in P1 vs P2 on outcomes in P3. From a practical perspective, it is fortunate that the transition from M in P1 to S in P2 did not increase negative outcomes relative to remaining in S from P1 to P2, given that removal of enrichments can have negative outcomes in some contexts [66–68]. Nevertheless, while it might be easier to provide resource choice for only a limited period during rearing, our findings support continued provision of choice throughout the whole rearing phase to maximise the welfare and fitness-related benefits of a choice environment.

Additional research is needed to investigate responses to resource variants other than those used in the current study, and to examine the generalisability of the findings to other animal housing systems. We have made observations at the pen level but, in future studies, it would be fruitful to investigate how different individuals within groups respond to multiple resource variants according to their personal preferences. Furthermore, for practical implementation, it will be necessary to establish optimal levels of accessibility to different resource variants (e.g. area or quantity/animal, and frequency of replenishment if ephemeral) under commercial conditions (i.e. large flocks) to avoid adverse effects due to resource competition. However, we did not find any indication of increased resource competition in the M treatment based on the methods of presenting resource choice in the current study.

5. Conclusions

Although much research has been conducted on effects of adding environmental enrichment materials and providing environments varying in structural complexity, few prior studies have focused on the long-term effects of providing domestic animals with resource choice. While resource choice implies environmental enrichment and increased environmental complexity, we hypothesised that early life exposure to resource choice itself has benefits. This could occur, for example, by stimulating learning to make fitness-promoting decisions about which substrate to use in different behavioural contexts, by providing early experience with diverse environmental stimuli such that it is easier to cope with subsequent environmental change, and by allowing expression of individual preferences that deviate from the group average. In support of our hypothesis, our findings indicate that the opportunity to choose between several types of litters and perches during rearing (P1 or P2) was mostly associated with higher levels of positively-valenced (pleasurable) behaviour and lower levels of negatively-valenced (aversive) behaviour, with some carry-over effects into adulthood (P3). Furthermore, these behavioural differences were correlated with long-term fitness-related health attributes including better feather condition and greater growth in P3, suggesting that early provision of a multi-choice environment is beneficial for adaptability and future quality of life. We conclude that exposure to multiple resource variants in the environment during rearing had long-term fitness-enhancing effects on behavioural development, promoting positive effects on laying hen welfare. In addition to contributing to fundamental understanding about developmental biology, these findings are of practical significance for animal production.

Competing interests

The authors declare no competing interests.

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Data availability

Pen-level data are provided in Supplementary Table S8, and individual-level data can be found in Supplementary Table S9.

Author contributions

All authors contributed to planning, RVH and LS collected the data, RVH conducted the statistical analyses and wrote the first draft of the manuscript. All authors contributed to revisions and approved the final draft.

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III Comb size, shape complexity and laterality of laying hens reared in environments varying in resource choice

Comb size, shape complexity and laterality of laying hens reared in environments varying in resource choice

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Abstract

The comb is an ornament involved in signalling condition in domestic fowl. We hypothesised that the comb size, comb shape complexity (i.e. rugosity, the jaggedness of the comb perimeter), and comb laterality of laying hens would be influenced by the degree of environmental enrichment experienced during juvenile development in the form of resource choice. In a 2 x 2 factorial crossover experiment, layer pullets in pens containing four perches of standard length and a standard litter area were exposed to single (S) *versus* multiple (M) choices of perch and litter types during Weeks 1-4 (Period 1) and/or Weeks 5-15 (Period 2) of rearing (n=4 pens/treatment combination: SS, SM, MS, MM) prior to transfer to standard laying pens for Weeks 16-27 (Period 3). Within (M) or across (S) pens, pullets were exposed to four perch types and four litter types. In Week 27, combs were photographed, and comb laterality (hanging on left or right side) was noted. Using a bespoke image analysis programme, we captured comb area (mm^2), perimeter length (mm), and rugosity ((perimeter length-horizontal length)/horizontal length) from comb photographs of 6-7 randomly selected hens/pen. We predicted that hens kept in the M environment during Periods 1 and 2 would have larger, more complex, and left-side-biased combs than those in the other treatment groups, reflecting reduced allostatic load. The predicted comb side bias was based on a possible bias in head posture/movements associated with greater right eye/ear use and left-brain hemispheric dominance. Contrary to our predictions, we detected an overall right-side bias in comb laterality, and no associations between resource choice treatment in Period 1 or 2 and comb area, perimeter length, rugosity, or laterality of the adult hens. Thus, variation in allostatic load resulting from the rearing treatments was insufficient to modify the trajectory of comb morphological development, possibly due to a ceiling effect when comparing environmental treatments on the positive end of the welfare spectrum. However, we did find that right-lopping combs had longer perimeters than left-lopping combs. Furthermore, among hens with right-lopping combs (“righties”), those with larger, longer-perimeter combs tended to be heavier, with less comb damage, while among “lefties”, those with larger, longer-perimeter combs were heavier and had less feather damage. In conclusion, comb characteristics were related to physical condition at the individual level but did not serve as sensitive integrated indicators of hen welfare in response to basic *versus* enhanced resource choice during rearing.

Keywords: chicken rearing, environmental enrichment, positive animal welfare, comb development, condition-dependent signalling

Implications

Laying hen combs are considered to function as condition-dependent signals of an individual’s fitness, meaning comb quality should be enhanced under more beneficial environmental conditions. We expected hens raised in more diverse environments, with different perch and litter types, to have larger, more complex combs than counterparts raised in environments more similar to commercial production systems (one perch- and litter type). However, although comb measures reflected physical condition, the treatments had no effect on comb development. Our results are relevant for poultry husbandry by showing that comb characteristics were not sensitive enough to differentiate between good *versus* better rearing conditions.

Introduction

Biological ornaments are conspicuous traits of animals that are considered to function as condition-dependent signals of mate quality during mate selection (Hill, 2014; Winters, 2018) though they may also serve a dual role as armaments (i.e. weapons or status badges; Berglund et al., 1996). Examples of visual ornamentation can be found in diurnal animals across many taxa, such as the colourful iridescent tail feathers of peacocks (*Pavo cristatus*; Dakin and Montgomerie, 2013) and the long eye stalks of stalk-eyed flies (*Teleopsis dalmanni;* Cotton et al., 2010). If ornaments are condition-dependent signals, ornament quality should be higher under more favourable environmental conditions. Zuk et al. (1990) reported such a difference in the head ornament size of unparasitised *versus* parasitised male red jungle fowl (*Gallus gallus,* progenitor of the domestic fowl). When ornamentation occurs in both sexes (monomorphic ornamentation), male ornaments are generally more exaggerated than those of females. Nevertheless, female ornaments can also provide information about their bearer's physical condition and reproductive potential (Nolazco et al., 2022), which is relevant for reciprocal mate selection and female resource competition (Kraaijeveld et al., 2007; Fitzpatrick and Servedio, 2018). For example, in choice tests, dominant feral domestic fowl males were found to mate sooner with females bearing relatively large head ornaments and to deposit more sperm when mating with them (Cornwallis and Birkhead, 2007).

Allostatic load can be defined as cumulative "wear and tear" affecting body condition and brain function that results from repeated attempts to adapt to environmental challenges (McEwen, 1998). It derives from the continual adjustments made by bodily systems to maintain allostasis (i.e. optimal function in the face of changing demands; McEwen, 1998). Individuals vary in their accrual of allostatic load, even in response to similar stressors, due to differences in genetics and in how they perceive their environment (Korte et al., 2005). Allostatic load can result from overworked allostatic systems due to frequent stress, failure of stress-related systems to shut off even when a response is successful, or unsuccessful stress responses that cause other physiological systems to overreact (McEwen, 1998). Allostatic load may affect the elaboration of ornamentation, such that adult ornamentation serves as an integrated signal of life-to-date stress resilience and quality of life. Larger, more complex ornaments may therefore indicate better welfare earlier in life.

The fleshy head ornament of domestic fowl and red jungle fowl is referred to as a comb. While the size of the domestic fowl comb is heritable, it also exhibits considerable individual variation (Shen et al., 2016), which is consistent with functioning as a signal of individual fitness. In laying hens, comb features such as comb size and colour have been connected to social status (e.g. O’Connor et al., 2011; Siegel and Dudley, 1963) and commercially important fitness-related measures such as body weight (e.g. Tufvesson et al., 1999) and fecundity (Wright et al., 2012). The comb is rudimentary at hatch but develops during sexual maturation under the influence of androgen hormones (Mukhtar and Khan, 2012), beginning about 8 weeks before the onset of lay in laying pullets (Eitan et al., 1998). Consequently, comb growth will be impacted by any stress-related variation in the circulating androgen levels of females, especially during the period leading up to the onset of lay.

Comb types vary across strains of domestic fowl, with the “single” comb type being typical of strains reared for commercial egg production. As single combs grow, they reach a point where they can no longer stand erect but start to tilt and eventually hang habitually (lop) on the right or left side of the head (Tufvesson et al., 1999; Wan et al., 2018). Mueller and Hutt (1942) reported that right-lopped combs were more common than left-lopped combs and noted that neither left-lopped fathers nor mothers consistently produced more left-lopped offspring. This finding suggests that comb laterality is influenced by environmental conditions during development. It is conceivable that comb laterality is related to individual differences in head posture and movements. The chicken brain shows lateralisation in the processing of sensory, social and unfamiliar stimuli, with lateralisation in the use of the eyes, ears and nares when evaluating environmental stimuli (Rogers, 2023). In particular, there is a bias towards using the left eye when evaluating novel and potentially dangerous visual stimuli (Rogers, 2010). Thus, more anxious birds, that can be expected to accumulate higher allostatic load, may more frequently evaluate their environment using their left eye and ear than calmer chickens, potentially increasing the likelihood of developing a right-lopped comb.

Chicken combs develop into intriguing, complex shapes. The comb shape varies in “jaggedness” of the outer comb-line (rugosity) between individuals, with differences in the number, width, and height of the points. After puberty, comb shape appears to remain relatively stable over time, consistent with a role in individual recognition (Guhl and Ortman, 1953). Genes associated with comb shape have been identified (Bakovic et al., 2022). However, comb shape can be altered by environmental factors such as frostbite, accidental tearing and pecking injuries, as well as by artificial comb dubbing (Marks et al., 1960). It is also possible that environmental conditions during rearing play a role in sculpting adult comb shape complexity as well as influencing comb size.

Commercial laying hens are commonly reared in an aviary system where, as chicks, they are kept in cage-like compartments in the first 4-8 weeks, after which the compartments are opened and the whole aviary including the litter floor becomes accessible. When the pullets are around 16 weeks of age, they are moved to an aviary house optimised for egg production, where they begin to lay eggs around 18-22 weeks of age and remain throughout adulthood. Exposure to environmental changes presents challenges (e.g. Brantsæter et al., 2016) that may contribute to allostatic load. Providing a more complex and diverse environment during juvenile development, both in the period prior to opening of rearing compartments and the period prior to the move to adult housing, may improve the birds' ability to adapt to environmental changes, minimising allostatic load and enhancing their ability to grow an elaborate comb. In support of this hypothesis, Nazar et al. (2022) and Skånberg et al. (2023) found that laying hen chicks reared for 3-4 weeks with access to multiple variants of perches and litter were less fearful and had greater adaptability when exposed to environmental change compared to chicks that were kept with only a single variant of each resource type.

A subsequent longer-term cross-over experiment investigated effects of resource choice during the chick (Period 1) and pullet (Period 2) rearing stages on outcomes in early adulthood (Period 3) when all birds were kept in the same environment (Holt et al., 2023a). Hens were reared with one perch type and one litter type (single-choice, representing a basic level of environmental enrichment), or four variants of perches and litter (multi-choice, representing an enhanced level of environmental enrichment), either throughout both Periods 1 and 2 or in succession with order counterbalanced across groups. Hens reared in the multi-choice environment in Period 2 exhibited higher body weights in Period 3 and less feather damage (related to more dust bathing and less severe feather pecking) in Periods 2 and 3 than those reared in the single-choice environment. Further, hens reared in the multi-choice condition in Period 1 performed more play in Period 1 and less aggressive pecking in Period 3 (Holt et al., 2023a). These findings suggest that allostatic load across Periods 1-3 was lower in multi-choice than single-choice hens. To investigate this potential effect of increased choice during rearing on comb development, the current study utilized comb data collected from the adult hens at the end of Period 3, coinciding with peak daily egg production.

We hypothesised that comb development would be differentially affected by the amount of resource choice experienced during rearing. Specifically, based on findings regarding plumage condition, growth, and aggressive pecking, we predicted that hens kept in the multi-choice environment during Periods 1 and 2 would have larger, more complex, and more left-side-biased combs at the end of Period 3, reflecting lower allostatic load, compared to hens kept in the single-choice environment in both periods. The design of our experiment also allowed us to investigate whether the timing and order of exposure to a multi-choice environment (Period 1 or 2) would differentially influence comb development. While we expected that exposure to the multi-choice environment in either period would result in more elaborated combs than single-choice alone, we predicted that a change from the single-choice to the multi-choice environment would have a positive impact on comb development, compared to a change in the reverse direction, as it could facilitate a spurt in comb growth associated with sexual maturation starting in Period 2. In addition to treatment effects, we also investigated variation in comb traits in relation to individual physical condition. We expected to find positive correlations between comb size metrics, comb shape complexity and body weight of individual hens, and negative correlations of these variables with feather and comb damage scores. Furthermore, we predicted that hens with left lopped combs (“lefties”) would be heavier, with larger, more complex combs and lower feather and comb damage scores, than hens with right lopped combs (“righties”).

Material and methods

Animal, housing and management

We conducted the study at the Swedish Livestock Research Centre of the Swedish University of Agricultural Sciences in Uppsala, SE. We obtained day-old laying hen chicks (Bovans Robust chicks, n = 364) with intact beaks from a local hatchery. We assigned them to 16 visually-isolated rearing pens (245 x 120 x 180 cm) in groups of 22-23 birds (balanced for group weight). Each pen contained a drinker line with four water nipples, two circular chick feeders, four

120-cm-long perches, and four shallow trays containing litter that were emptied and refilled at least once weekly as needed to maintain their contents. In Week 2, we replaced the chick feeders with a round hanging feed hopper. We increased the perch height from 15 to 45 cm in Week 2 and to 55 cm in Week 5, when we exchanged the shallow litter trays for deeper trays. We gave *ad libitum* access to water and standard starter feed for the first 6 weeks, followed by standard growing feed. The room temperature was set at 25 °C for the first 9 weeks, and then dropped to 20 °C. A hanging heat lamp provided additional warmth at chick level for the first 4 weeks. The photoperiod was 20 h on Day 1 and gradually reduced to 10 h by Week 6. Mean light intensity was 18 lux at bird level, except for a 15-min dawn and dusk period at the beginning and end of each photoperiod.

In Week 16, we transferred the birds in their groups to 16 adult laying pens (362 x 356 x 297 cm). These pens had a 132 x 356 cm solid-floored area and three narrow platforms along one wall. They also had a raised slatted area (230 x 356 cm) providing access to two colony nests, two hanging feed hoppers, a bell drinker, five elevated perches, and five low perches attached to the slats. Room temperature was 20 °C and light intensity at hen level averaged 5.4 lux. The photoperiod was 10 h in Weeks 16-19, then increased it by one hour weekly to 14 h. At the end of the experiment (Week 27), we adopted the hens out to local poultry keepers.

Experimental design

The study was divided into three experimental periods. In Period 1 (Weeks 1-4, chick rearing period), we assigned chicks to one of two treatments (Fig. 1a) in a randomised block design: Single-choice or Multi-choice. Single-choice pens (n = 8) had four perches of the same type (a wooden plank, a rope comprising three horse leading reins braided together, a round rubber perch or a wire mesh perch) and one litter type in all four litter trays (sand with particle diameter ≤ 0.03 mm, wood shavings, straw, or peat). We formed four unique perch-litter combinations that were balanced across the Single-choice pens (Fig. 1b). Each Multi-choice pen (n= 8) had all four different perch types and all four different litter types (one litter type/tray). We balanced the location of each resource type within the pen across the Multi-choice pens. In Period 2 (Weeks 5-15, pullet rearing period), we switched the treatment in half the pens (n= 4 pens/treatment) to the opposite treatment (Fig. 1). In Period 3 (Weeks 16-27, adult laying period), all groups were housed in standard laying pens with resource types not experienced during rearing. These included crushed straw pellets as litter and a variety of wooden and plastic perches of novel dimensions. See Holt et al. (2023a) for further details regarding the experimental conditions.

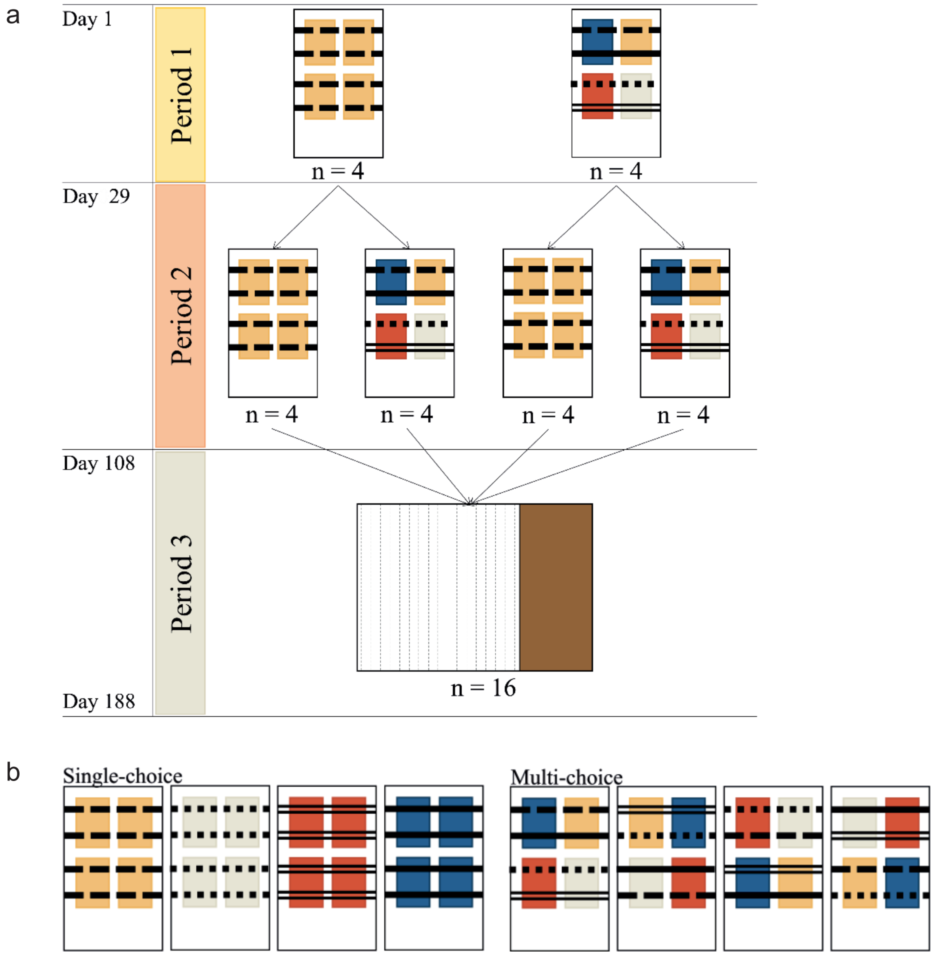

Fig. 1. Experimental design. a) Experimental timeline. In Period 1 (Days 1-28), groups (n=16) of laying hen chicks were assigned to one of two treatments: Single-choice, with one of four possible perch types (black lines) and litter types (coloured rectangles), or Multi-choice, with all four perch and litter types. In Period 2 (Days 29-107), half the groups were switched to the opposite treatment. In Single-choice, the type of perch and litter was balanced across replicate pens, and in Multi-choice, the location of each perch and litter type was balanced across replicate pens. All groups were moved to similar pens for Period 3 (Days 108-188) that consisted of a slatted floor with several novel perches (white rectangle) and a large litter area with one novel litter type (brown rectangle).
b) Exemplars of pens on the Single-choice and Multi-choice treatments.

Data collection

We collected data at the end of Period 3 (Days 184-186). Each hen was weighed and feather and comb scored individually, and the side of the head to which her comb lopped (right or left, i.e. comb laterality) was noted. One researcher then laid the bird on her side on a table with her comb (inner side facing up) lying on a black clipboard with attached ruler while another photographed the comb, with the ruler in view to provide the scale. After excluding 10 photographs with blurry or buckled comb images, we extracted comb measurements (Table 1) from the photographs of 6 to 7 randomly selected birds per pen (100 birds in total, based on power analysis to calculate the number of comb pictures required to reach power > 0.80). We determined the maximum length and height of each comb, as well as the area and perimeter length of the comb, using a custom-made image analysis programme written in Linux (Ubuntu 20.04; Canonical Ltd., 2020). Scaling was based on the number of pixels along a 10-mm length of the ruler in the image. As a measure of comb shape complexity controlling for comb size, we calculated a rugosity index by subtracting the comb length from the perimeter length and dividing the difference by the comb length (Table 1). This measure equates to the classical assessment of rugosity by draping a flexible transect line over an uneven surface and calculating its length relative to the flat distance between the two endpoints.

Table 1. Comb and physical condition measures taken from individual laying hens (n=100) at 184-186 days of age (end of Period 3), with overall means with SE.

Variable	Description	Mean	SE
Comb traits			
Comb length (mm)	The longest horizontal length of the comb parallel to a line from the front of the head to the back of head	85.2	0.81
Comb height (mm)	The greatest vertical height of the comb from the top of the head to the tip of the highest comb peak, perpendicular to the comb length.	47.6	0.65
Comb area (mm^2)	The two-dimensional surface area of the inner side of the comb (side adjacent to the head).	2359.0	44.70
Comb perimeter length (mm)	Continuous line tracing the complete boundary of the comb, providing a combined measure of comb size and shape.	320.0	4.50
Comb shape complexity	Index of rugosity calculated as (comb perimeter–comb length)/comb length, representing shape controlling for size.	2.8	0.04
Comb laterality (n of birds)	Lop of the comb to the left or right side of the head.	24 left	76 right
Physical condition measures			
Body weight (g)	Individual weight to nearest g.	1579.7	10.87
Feather damage score (0-4)	Proportion of scruffy, split, broken or missing feathers, scored as 0 (0 %), 1 (1-25 %), 2 (25-49 %), 3 (50-74 %) or 4 (75-100 %) on each of six body regions (head, neck, wings, rump, belly, tail), averaged to obtain a mean score.	1.3	0.05
Comb damage score (1-3)	Number of peck wounds on the comb, scored as 1 (0-3), 2 (4-6) or 3 (>6).	2.2	0.07

Quality assurance

All comb data were collected by one observer. To assess interobserver concordance, a second observer used the same custom-made image analysis programme to extract comb area and comb perimeter data from 16 birds (one randomly-selected bird/pen; four birds/treatment). The results indicated good agreement between observers (mean intraclass correlation=0.88, calculated using the package psych; Revelle, 2023). To validate our custom-made image analysis programme, we compared the comb area and perimeter results from the 16 birds with results from the same birds obtained using the established programme, ImageJ (Schindelin et al., 2012). The mean intraclass correlation was 0.93, indicating high reliability.

Statistical analysis

Statistical analyses were conducted in RStudio (RStudio Team, 2020) using R 4.3.1 (R Core Team, 2023), with alpha=0.05. Model fit was examined and confirmed using residual diagnostics plots produced by the DHARMa package (Hartig, 2022), and confidence intervals and estimated marginal means were obtained using the emmeans package (Lenth et al., 2023). To examine treatment effects, we evaluated the impact of Period 1 treatment, Period 2 treatment and their interaction on the comb variables: comb area, comb perimeter length, comb shape complexity and comb laterality. We did not include comb length and height in these analyses to avoid redundancy as these variables were closely related to comb area and perimeter. As birds were kept in groups, we included pen as a random effect, whereas experimental block had no effects and was excluded from all models. Continuous variables were analysed using linear mixed models (lme4 package; Bates et al., 2015) with Gaussian distribution, fitted with restricted maximum likelihood. T-tests for these models used the Satterthwaite approximation for degrees of freedom. We analysed comb laterality, a nominal variable, using a generalised linear mixed model (lmerTest package; Kuznetsova et al., 2017) with binomial distribution, maximum likelihood parameter estimation and Laplace approximation.

A chi-square test was used to assess whether combs lopped more to one side than expected by chance. Linear mixed models were used to compare comb measurements, body weight and feather damage scores of hens with right-*versus* left-lopped combs, while their comb damage scores were compared using a cumulative link mixed model with maximum likelihood parameter estimation and Laplace approximation (ordinal package; Christensen, 2022; RVAideMemoire package; Hervé, 2023). To evaluate associations between the variables for “righties” and “lefties”, Pearson correlations between the comb traits, body weight and feather and comb damage scores were calculated.

Results

Comb response to resource choice

None of the analysed comb variables were significantly affected by the treatments experienced during Period 1, Period 2 or their interaction (Table 2).

Table 2. Estimates with 95% confidence interval (CI) from linear mixed models (T statistic) and, for comb laterality, odds ratios with 95% CI from a generalised linear mixed model (Z statistic) evaluating associations of treatment (single- *versus* multiple choices of perch and litter types) and period (Period 1: Week 1-4, Period 2: Week 5-15) with comb measures of 184-186-day-old laying hens (n=100). The single-choice treatment was the reference treatment. See Table 1 for variable definitions.

		Predictor		
Response variable	**Statistic**	**Period 1**	**Period 2**	**Periods 1 × 2**
Comb area	*Estimate*	139.5	85.6	-220.1
	95 % CI	-113.22 – 392.18	-167.15 – 338.25	-577.45 – 137.29
	T	1.10	0.67	-1.22
	Df	96	96	96
	P	0.276	0.503	0.224
Comb perimeter	*Estimate*	-4.2	4.2	2.4
	95 % CI	-29.80 – 21.41	-21.42 – 29.79	-33.77 – 38.65
	T	-0.33	0.32	0.13
	Df	96	96	96
	P	0.746	0.746	0.894
Comb shape complexity	*Estimate*	-0.1	0.1	-0.0
	95 % CI	-0.32 – 0.17	-0.17 – 0.32	-0.35 – 0.34
	T	-0.63	0.62	-0.03
	Df	12.5	12.5	12.5
	P	0.539	0.545	0.979
Comb latera-lity	*Odds ratio*	0.7	1.2	2.7
	95 % CI	0.12 – 4.37	0.22 – 6.70	0.23 – 30.07
	Z	-0.33	0.22	0.79
	Df	Infinity	Infinity	Infinity
	P	0.739	0.825	0.431

Comb laterality

Of the 100 birds sampled, 76 hens were "righties" while the remaining 24 were "lefties" (Fig. 2; Supplementary Fig. S1). This right-side bias was greater than that expected in the absence of laterality (i.e. with a 50% chance of the comb lopping in either direction; χ^2=13.52, df=1, *P*<0.001). "Righties" had combs with longer perimeters than "lefties" ($t_{98.0}$=2.24; P=0.028; Fig. 3) and tended to have combs with greater shape complexity ($t_{96.9}$=1.94; P=0.055). Results for the remaining variables did not differ between "righties" and "lefties" (see Table 1 for overall means with SE; Supplementary Table S1 for estimates).

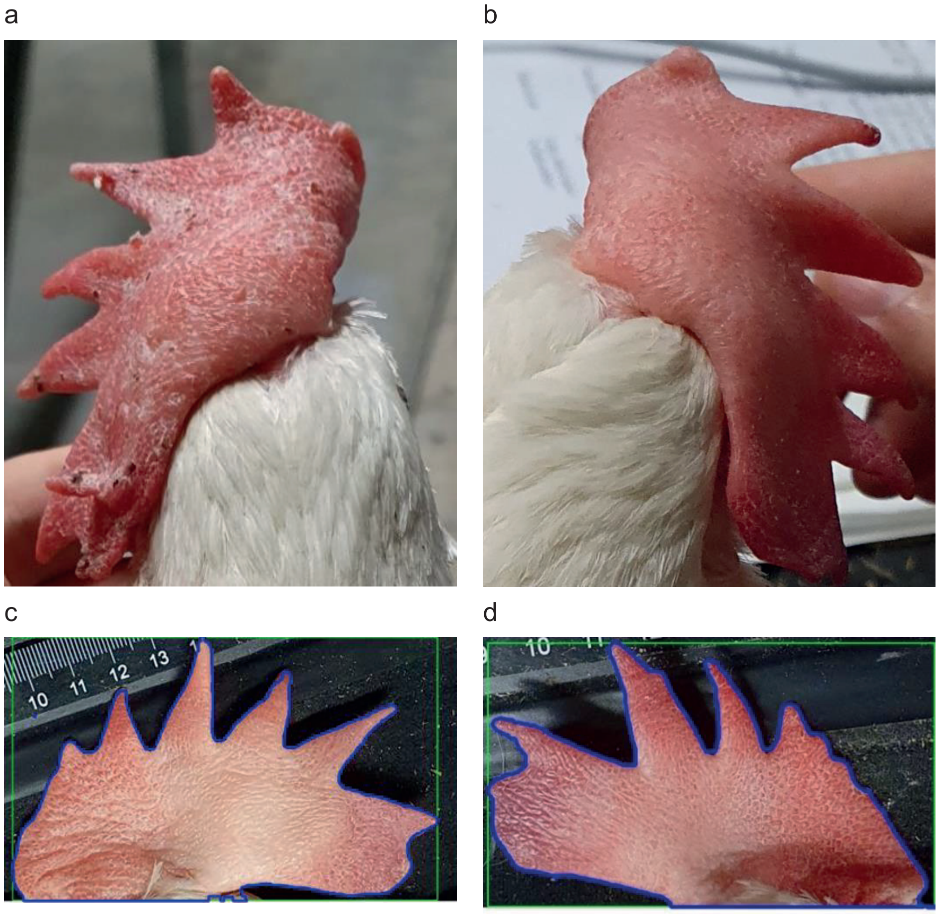

Fig. 2. Laying hen combs at 184-186 days of age: a) a hen with a left-lopped comb; b) a hen with a right-lopped comb; c-d) examples of outputs from a bespoke image analysis programme showing measurement of comb length and height (green box), and comb area and perimeter length (blue outline), of the inner surface of a left-lopped comb (c) and a right-lopped comb (d) based on the picture scale (n pixels/10 mm). See Supplementary Fig. S1 for uncropped photographs with further details.

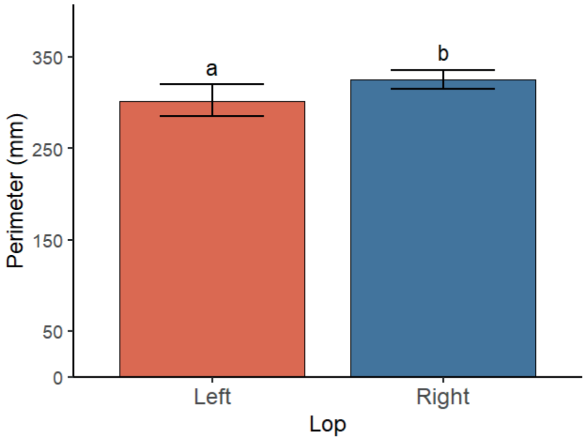

Fig. 3. Comb perimeter length (mm) of hens with left- *versus* right-lopping combs (mean with 95% confidence interval; a, b indicate difference at $P<0.05$).

Comb trait correlations

Comb perimeter was correlated with comb area (“righties” and “lefties”: $P<0.001$) and comb shape complexity (“righties”: $P<0.001$; “lefties”: $P=0.010$; Fig. 4). Both comb area (“righties”: $P=0.056$; “lefties”: $P=0.021$) and comb perimeter length (“righties”: $P=0.022$; “lefties”: $P=0.085$) were weakly correlated with body weight. In “lefties”, heavier hens ($P=0.036$) with bigger comb areas ($P=0.013$) and longer comb perimeters ($P=0.042$) sustained less feather damage whereas among “righties”, hens with bigger comb areas ($P=0.082$) and longer perimeters ($P=0.052$) tended to have less comb damage.

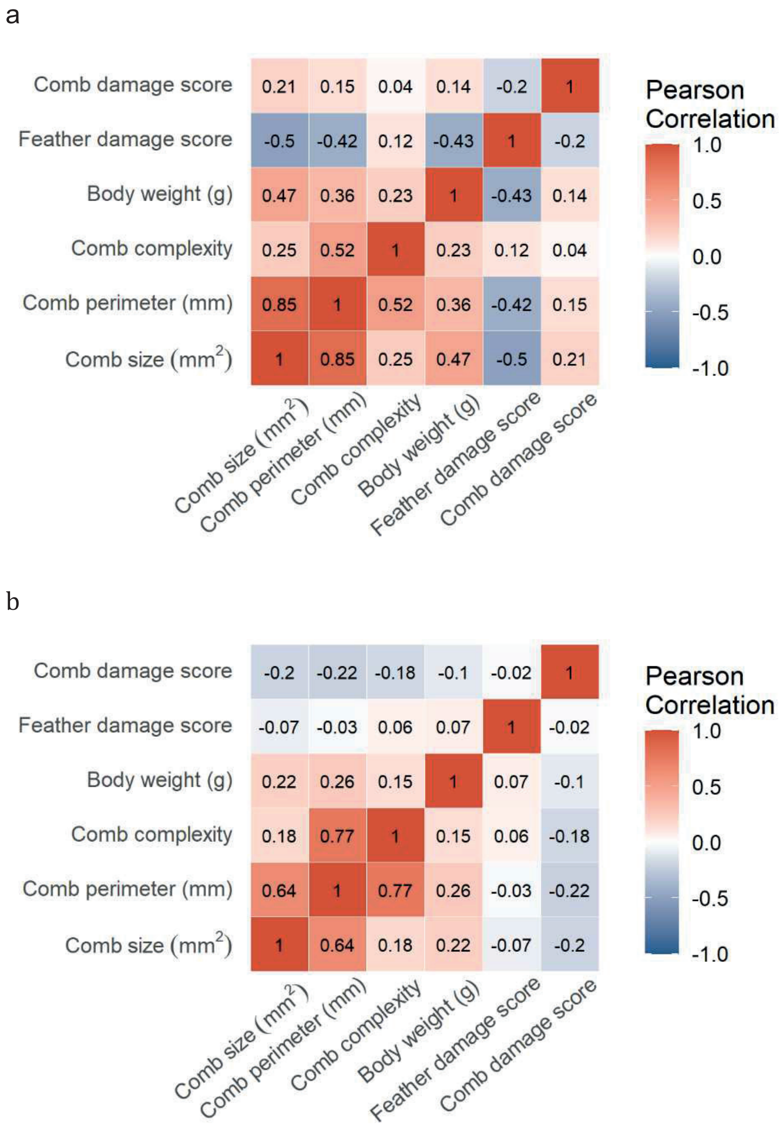

Fig. 4. Heatmaps of correlations (*r* values) between comb and physical condition variables of laying hens at 184-186 days age; with values for hens with (a) left-lopping combs (n=76) and (b) right-lopping combs (n=24).

Discussion

Stressors experienced during development, such as those related to exposure to a novel environmental may increase allostatic load via the hypothalamic-pituitary-adrenal axis and inhibit androgen production, thereby limiting the development of condition-dependent signals such as the comb of laying hens. Providing laying hens with resource choices during rearing, thereby increasing the degree of environmental enrichment and allowing for more learning opportunities, may buffer against the build-up of allostatic load by creating more adaptable, stress-resilient hens. On this basis, we predicted that hens reared in the more complex Multi-choice environment would develop larger, more complex, and left-side-biased combs compared to those reared in the less complex Single-choice environment. However, we did not detect significant differences in any of the comb measures between hens kept in the Multi-choice *versus* the Single-choice environment. There was neither an additive effect of the duration of exposure to the Multi-choice *versus* Single-choice environment nor an interactive effect related to the order of exposure to these environments. Thus, it appears that treatment-related differences in allostatic load were too small to produce consistent differences in comb traits. Both environments provided plentiful resources and space per bird and, although the Multi-choice treatment had more beneficial effects on behaviour and body condition (Holt et al., 2023a), the Single-choice treatment provided basic enrichments including access to multiple perches and frequently refreshed litter. Our results suggest that our treatment comparison was operating on the positive end of the welfare spectrum, where a ceiling effect may have limited treatment differences in the morphological development of laying hen combs.

The lack of treatment effect on comb laterality may have been influenced by incubation in the dark, as is typical at commercial hatcheries. Although some lateralisation of the brain occurs in dark-incubated chicks, it is reduced relative to that of chicks exposed to light during incubation (Rogers, 2023). It is also possible that use of different hemifields of the eyes for investigating different types of environmental stimuli (Vallortigara et al., 2001) reduced overall laterality of head movements over the course of comb development, reducing the impact of the post-hatch environment on comb laterality. Nevertheless, we observed a significant overall right-side comb bias in our hens as also reported by Mueller and Hutt (1942). Perhaps this outcome reflected the reported greater fearfulness of chicks incubated in the dark (Archer and Mench, 2017). Fearful chicks favour left eye use over right eye use (Rogers, 2010), which may have led to the preponderance of “righties”. Because the “lefties” were relatively rare, they may have been more likely to be harassed by other hens, leading to their shorter comb perimeters and a tendency for lower comb shape complexity, although the lack of other systematic differences between the “righties” and “lefties” argues against this explanation. On a practical note, after combs have lopped to one side or the other, their continued growth can lead to obstruction of vision on that side, which may have implications for how hens perceive their surroundings. Large pendulous combs may impair net welfare unless their excessive size provides compensatory benefits such as improved thermoregulation in hot climates.

At the individual level, we detected a weak correlation between body weight and both comb area and comb perimeter length of hens in Week 27, when egg production was peaking. This finding is consistent with other studies reporting a correlation between body weight and comb size in laying hens (e.g. Tufvesson et al., 1999). However, not all studies have detected a correlation (e.g. Wright et al., 2012). Wan et al. (2018) found a correlation at 24 weeks of age, but not before or after this age, when comparing different breeds of laying hens between the ages of 4-30 weeks. These findings suggest that body weight and comb size may be more tightly linked when comb growth is maximal (around puberty), especially in males given that they grow faster and larger than females. It can also be expected that the correlations between body weight, comb area and comb perimeter length manifest more strongly under conditions when welfare is compromised by prolonged undernutrition, such as when food intake is limited due to competition, disease or natural incubation of eggs, or when food reserves are drained by adverse weather conditions or parasitism (e.g. Zuk et al., 1990). This was not the case in our healthy population of young hens with *ad libitum* access to food and other resources, where the hens' opportunity to reach their genetic potential for egg production was high. We also note that relationships between comb traits and reproductive outcomes can vary between selection lines (McGary et al., 2003).

We explored associations between plumage condition and comb measures as Holt et al. (2023a) found that, at the group level, birds exposed to the Multi-choice environment had less feather damage than birds exposed to the Single-choice environment, which was associated with a lower level of severe feather pecking behaviour in Periods 1 and 2, and a higher rate of dustbathing in Periods 2 and 3. We found a negative relationship between the mean feather damage scores of individuals and their comb area and perimeter length, but only in the less common “lefties”. Overall, the differences in feather damage were relatively minor, being mainly due to differences in feather scruffiness, with limited feather splitting or breakage and no observations of heavy feather loss from any of the five evaluated body regions. Our results for “righties” are consistent with those of Tahamtani et al. (2017) who found no difference in laying hen comb size between feather peckers, their victims, or control hens that were in neither of these categories. Although we did not find an association between the resource choice treatments and comb damage (Holt et al., 2023a), among the “righties”, those with bigger comb areas and longer comb perimeters tended to have less comb damage. This finding is in keeping with evidence that laying hens with larger combs are more likely to win agonistic encounters and maintain a higher social status than hens with smaller combs (e.g. Marks et al., 1960; Siegel and Dudley, 1963; Cloutier and Newberry, 2000; O’Connor et al., 2011).

While we detected some correlations between morphological comb traits and indicators of physical condition, other more rapidly varying comb characteristics may be better suited as welfare indicators, especially over shorter periods. For example, Ross et al. (2020) used the decrease in comb temperature and latency to return to basal comb temperature as measures of stress resilience. Comb colour can be another useful short-term welfare indicator (e.g. Zuk et al., 1990) if reliably measured without disturbing the birds.

Conclusions

Although the Multi-choice condition promoted adaptability to the adult environment as indicated by improved adult plumage condition, increased body weight and lower aggression (Holt et al. 2023a), the treatments did not modify the developmental trajectory of the comb. The lack of treatment differences in the measured comb traits argues against their use as reliable integrative indicators of the impact of rearing conditions on hen welfare when comparing results from good environments with even better environments (i.e. in studies of positive welfare), as done in this study. Providing multiple resource choices would, nevertheless, be beneficial for accommodating individual differences and adding complexity and diversity to housing environments.

Ethics approval

This study was performed on a common production species, typically kept indoors under human care. We kept the birds in small, stable groups at low density throughout the experiment (Periods 1 and 2: minimum 1,278.3 cm^2/bird, Period 3: minimum 5,603.1 cm^2/bird). All birds were familiar with the close proximity of, and handling by, the involved personnel. All procedures were approved by the Uppsala Animal Experiment Ethics Board (Number 5.8.18-11549/2017).

Data and model availability

The data and R code used to produce the statistics and figures presented in this article are publicly available in Github: https://github.com/RVHolt/ChoiceCombs.git (Holt et al. 2023b). Access to the bespoke image analysis programme may be given upon request.

Generative AI and AI-assisted technologies

The authors did not use any artificial intelligence assisted technologies in the writing process.

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Declaration of interest

None.

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8 Appendices

Supplementary Information for paper II:

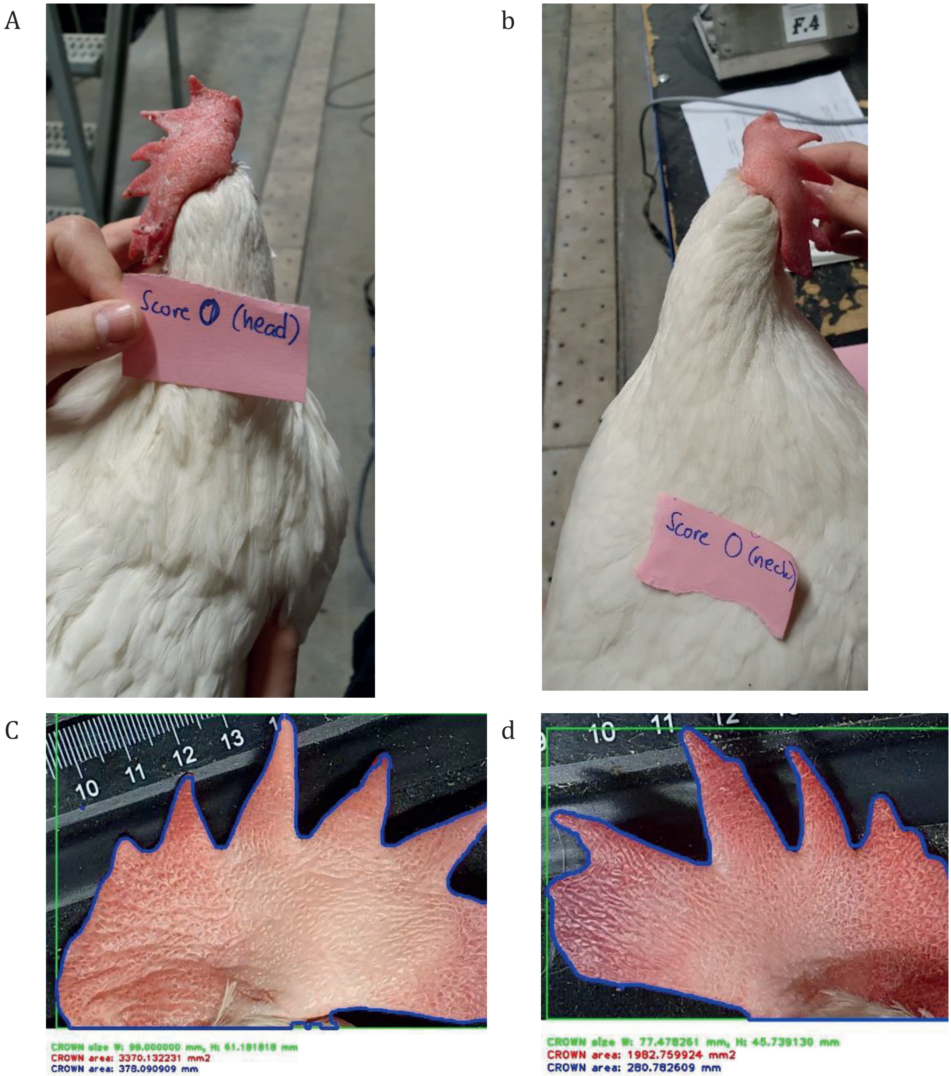
Resource choice during juvenile development contributes to long-term welfare in laying hens

Due to the size and number of supplementary materials for this paper, please see the excel file at https://github.com/RVHolt/LongtermChoice.git

Supplementary Information for paper III:

Comb size, shape complexity and laterality of laying hens reared in environments varying in resource choice

Supplementary Figure S1



```
CROWN size W: 99.000000 mm, H: 61.181818 mm
CROWN area: 3370.132231 mm2
CROWN area: 378.090909 mm
```

```
CROWN size W: 77.478261 mm, H: 45.739130 mm
CROWN area: 1982.759924 mm2
CROWN area: 280.782609 mm
```

Supplementary Figure S1. Original, uncropped pictures from Fig. 2. a) left-lopped comb with a score of 0 for feather damage in the head region. b) right-lopped comb with a score of 0 for feather damage in the neck region. c-d) outputs of the comb measuring algorithm of the bespoke image analysis program.

Supplementary Table S1

Supplementary Table S1. Associations of comb laterality (right- vs left-lopped comb) with comb and physical condition measures of 184-186-day-old laying hens (n=100), showing estimates with SE from either linear mixed models (T statistic) or a cumulative link mixed model (for comb damage score, Z statistic). Hens with right-lopped combs formed the reference group. See Table 1 for variable definitions.

Response variable	Estimate	SE	*df*	*T* or *Z*	*P*
Comb size (mm^2)	-46.17	105.080	98.0	-0.44	0.661
Comb perimeter (mm)	-23.11	10.332	98.0	-2.24	0.028
Comb shape complexity	-0.17	0.087	96.9	-1.94	0.055
Body weight (g)	-18.44	25.160	97.3	-0.73	0.466
Feather damage score (0-4)	-0.02	0.090	91.6	-0.20	0.845
Comb damage score (1-3)	0.64	0.461	Infinity	1.38	0.168

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