UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

BEHAVIOR AND PRODUCTIVE INDICATORS FOR BROILER CHICKENS: IS ENVIRONMENTAL ENRICHMENT ALWAYS POSITIVE?

MARCONI ITALO LOURENÇO DA SILVA

Thesis submitted to the Graduate Program in Animal Sciences in fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Sciences

BOTUCATU, SP
August, 2023

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DOCTORAL THESIS

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Advisor: Assoc. Prof. Dr. Ibiara Correia de Lima Almeida Paz

Co-advisor: Assoc. Prof. Dr. Leonie Jacobs

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“There is no such thing as a neutral education process. Education either functions as an instrument that is used to facilitate the integration of generations into the logic of the present system and bring about conformity to it, or it becomes the "practice of freedom", the means by which men and women deal critically and creatively with reality and discover how to participate in the transformation of their world.”

(Paulo Freire)

BIOGRAPHY

Marconi Italo Lourenço da Silva is an Animal Scientist from Federal Rural University of Pernambuco, Brazil (2017). Master in Animal Science with an emphasis on poultry production, animal welfare, and ethology from Sao Paulo State University, campus in Botucatu, Brazil (2020). In His Ph.D. in Animal Science at the same institution, he developed studies related to the use of environmental enrichment aiming to increase the quality of life of chickens. Still during his Ph.D., Marconi performed a sandwich doctorate in a 15-month period at Virginia Tech under the supervision of Dr. Leonie Jacobs. At Dr. Jacobs' lab, Marconi performed studies on cognitive bias tests for broiler chickens.

DEDICATION

To my parents, Sandra Maria Santana da Silva and Ivanildo Lourenço da Silva (*in memoriam)*, for their support at any time, unconditionally. I am now finishing this graduate degree due to their effort to provide me with conditions and for always believing in me.

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COMPORTAMENTO E INDICADORES PRODUTIVOS PARA FRANGOS DE CORTE: O ENRIQUECIMENTO AMBIENTAL É SEMPRE POSITIVO?

RESUMO – Com o consenso que os animais são seres sencientes as pesquisas sobre o bem-estar animal estão focadas no indivíduo e suas experiências. Dois experimentos foram conduzidos, objetivou-se no Exp. 1, avaliar o uso de enriquecimento ambiental e a produtividade, comportamento, prevalência de espondilolistese em frangos de corte de crescimento rápido. No Exp. 2, objetivou-se desenvolver um teste de viés de julgamento (JBT) para frangos de corte de crescimento lento, utilizando os aspectos sociais da espécie, para avaliar os efeitos do medo, ansiedade e estresse crônico e mensurar os estados afetivos dos animais. No Exp. 1 foram utilizadas 2400 aves Ross® AP95, machos, com um dia de idade. Os animais foram alocados em 4 tratamentos distribuídos em delineamento experimental casualizado com 4 repetições cada. Os tratamentos foram: controle (C) - ambiente similar ao comercial sem enriquecimento; ambientes enriquecidos com feno (HB), plataformas com degraus (SP), ou projetores de luz (LL). No Exp. 2 foram utilizadas 600 aves, Hubbard Redbro, machos, com um dia de idade. Os animais foram alocados em 2 tratamentos distribuídos em delineamento em blocos casualizados, com 6 repetições cada. Os tratamentos foram: controle - baixa complexidade, similar aos padrões comerciais, ou alta complexidade, adição de enriquecimentos permanentes e temporários. No Exp. 1, quando criados com acesso a SP e LL, houve menor frequência de espondilolistese quando comparados aos frangos criados sem enriquecimento ou com acesso a HB. Frangos com acesso a SP apresentaram maior rendimento de asas e menos gordura abdominal comparados com o grupo C. Frangos com LL e HB exploraram mais e descansaram menos que os animais dos tratamentos C e SP. No Exp. 2, medo, ansiedade e estresse crônico não afetaram o JBT. Frangos aproximaram e bicaram mais as pistas ambíguas comparado com as pistas não reforçadas. Frangos do tratamento controle aproximaram mais rápido das pistas ambíguas do que os frangos do tratamento alta complexidade, sugerindo que eles estão em estados afetivos mais positivo. Frangos submetidos à alta complexidade foram mais estressados. Em conclusão, enriquecimento ambiental reduz a prevalência de espondilolistese e melhora o comportamento exploratório. O hiper enriquecimento ambiental do Exp. 2 não foi apropriado para frangos de corte de crescimento lento.

Palavras-chave: Avicultura, comportamento, cognição, espondilolistese, estados afetivos, performance.

BEHAVIOR AND PRODUCTIVE INDICATORS FOR BROILER CHICKENS: IS ENVIRONMENTAL ENRICHMENT ALWAYS POSITIVE?

ABSTRACT - With the consensus that animals are sentient beings, research on animal welfare is focused on the individual and its experiences. Two experiments were conducted, the objective of Exp. 1 was to evaluate the use of environmental enrichment on the productivity, behavior, and prevalence of spondylolisthesis in fast-growing broilers. In Exp. 2, the objective was to develop a judgment bias test (JBT) for slow-growing broilers, using the social aspects of the species, to assess the effects of fear, anxiety, and chronic stress and to measure the affective states of the broilers. In Exp. 1, 2400 one-day-old, male, Ross® AP95 chicks were used. The animals were allocated into 4 treatments distributed in a randomized experimental design with 4 replications each. The treatments were: control (C) - environment similar to the commercial one without enrichment; environments enriched with hay (HB), step platforms (SP), or laser lights (LL). In Exp. 2, 600 one-day-old, male, Hubbard Redbro chicks were used. The animals were allocated into 2 treatments distributed in a randomized block design, with 6 replications each. Treatments were: low complexity, environment similar to commercial settings, or high complexity, a combination of permanent and temporary enrichments. In Exp. 1, when raised with access to SP and LL, there was a lower frequency of subclinical spondylolisthesis when compared to chickens raised without enrichment or with access to HB. Chickens with access to SP had a higher wing yield and less abdominal fat compared to the C group. Chickens with LL and HB explored more and rested less than the animals in the C and SP treatments. In Exp. 2, fear, anxiety, and chronic stress did not affect JBT training and testing performance. Chickens approached and pecked more the ambiguous cues near the reward cue than those near the neutral cue. Chickens in the control treatment approached the ambiguous cues faster than chickens in the high complexity treatment, suggesting that they are in more positive affective states. Chickens kept in high-complexity treatment were more stressed. In conclusion, environmental enrichment reduces the prevalence of spondylolisthesis and improves exploratory behavior. The enrichment strategy in Exp. 2 was not suitable for slow-growing broilers.

Keywords: Affective states, behavior, cognition, spondylolisthesis, performance, poultry.

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CHAPTER 1

1. INITIAL CONSIDERATIONS

Broiler chicken production in Brazil and the world has significantly evolved over recent years. With investments in technological innovations in genetic improvement, ambiance, nutrition, and health, broilers today are more efficient and productive (ABREU; ABREU, 2011). Brazilian production stands out on the world stage, being the largest exporter and third-largest chicken meat producer (ABPA, 2022). Such Brazilian importance in the world poultry market makes discussions related to the welfare of these chickens more relevant for maintaining the country's potential.

Lameness is the main welfare problem encountered in poultry production. Spondylolisthesis, for example, is a deformity in the sixth thoracic vertebra of chickens that compresses the spinal cord, causing pain and difficulties in walking, which can progress to paralysis of the pelvic limbs in clinical cases. (DINEV, 2013). The subclinical form of spondylolisthesis can affect 15 to 47% of the flock, silently compromising the welfare.

With the advancement of animal welfare science and the consensus that animals are sentient beings, research on animal welfare is increasingly focused on the individual and their experiences throughout raising (CARENZI; VERGA, 2009). Based on this, making the environment more complex and dynamic using environmental enrichment resources is a strategy adopted by researchers and industry to improve birds' quality of life in production systems.

Environmental enrichment is a management strategy in poultry barns that increases the expression of natural behaviors, meeting the natural living sphere from the animal welfare conceptualization (FRASER, 2008). Additionally, increased locomotion due to increased exploratory activity promoted by the environmental enrichment use strengthens the locomotor system and reduces the lameness prevalence (LOURENÇO DA SILVA et al., 2021). Thus, meeting the basic health and functioning sphere from the animal welfare conceptualization (FRASER, 2008). Even with a limited number of studies on broiler chickens, the use of environmental enrichment is associated with an increase in positive emotional experiences, improving the animals' affective states (ANDERSON et al., 2021a, 2021b) and, in turn, meeting the affective state sphere from the animal welfare conceptualization (FRASER, 2008).

The development and application of cognitive bias tests, such as the judgment bias test, by ethologists, has been shown to be a valuable tool to assess animal’s affective states (LAGISZ et al., 2020). However, they have limitations in the applicability for chickens, with long training phases and varied success rates, which may be related to how the test is designed and applied for the species. Therefore, studies focused on improving the test are necessary for the viability of this tool in animal welfare assessments. The effects of environmental enrichment on the prevalence of subclinical spondylolisthesis and a social-pair judgment bias test for broiler chickens are currently knowledge gaps in the literature.

This doctoral thesis evaluates the current understanding of the use of environmental enrichment in the production and welfare of broiler chickens. The literature review in **chapter 1** focuses on animal welfare parameters and how husbandry conditions, lameness, and environmental enrichment impact broiler welfare. In addition, the literature review dives into the current understanding of cognition, emotions, and affective states in broiler chickens. Additionally, two more chapters were included, which were published in high-impact scientific journals.

The study described in **chapter 2** entitled "Providing environmental enrichments can reduce subclinical spondylolisthesis prevalence without affecting performance in broiler chickens" investigates the environmental enrichment effects on the prevalence of subclinical spondylolisthesis and the performance of broiler chickens. This chapter is formatted according to the guide for authors of the journal PLoS ONE, where it was published. https://doi.org/10.1371/journal.pone.0284087

The study described in **chapter 3** entitled "Social-pair judgment bias testing in slow-growing broiler chickens raised in low- or high-complexity environments" investigates the environmental enrichment effects on the affective states of slow-growing broiler chickens. In addition, this study developed a new judgment bias test considering the social aspects of the species. This chapter is formatted according to the guide for authors of the journal Scientific Reports, where it was published. https://doi.org/10.1038/s41598-023-36275-1

Finally, the general implications of the studies developed in this thesis were included in **chapter 4.**

2. LITERATURE REVIEW

2.1 Animal welfare

The understanding of animal welfare has evolved over the years; animal welfare is related to the individual and their experiences throughout life (CARENZI; VERGA, 2009). Although multiple animals are raised under the same conditions, each individual may have a different level of welfare due to their life experience in the breeding environment, their health, and their genetics (BROWNING, 2022). Widely used since it was mentioned in the Brambell report (1965) and improved by Farm Animal Welfare Council (FAWC) in 1993, the concept of the five freedoms guided the building of legislation, policy statements on the subject, and standards related to human responsibility to ensure animal welfare (MELLOR, 2016), being them:

(1) Freedom from hunger and thirst
(2) Freedom from discomfort
(3) Freedom from pain, injury, or disease
(4) Freedom to express normal behavior
(5) Freedom from fear and distress

This concept has been internationally recognized as a fundamental principle of animal welfare (VAPNEK et al., 2010). With the consensus that animals are sentient beings, research on animal welfare is focused on the individual and its experiences, researchers began to understand that animal welfare goes far beyond physical issues and involves the animal's willingness (DAWKINS, 2017). Thus, researchers began to consider the positive emotions of animals, also known as positive welfare (MELLOR, 2016; LAWRENCE; VIGORS; SANDØE, 2019). Then comes a multifactorial and comprehensive approach to assessing the animal’s mental welfare, where the animal has a life worth living. (GREEN; MELLOR, 2011).

The animal welfare conceptualization involves 3 spheres that are interconnected (Figure 1), namely basic health and biological functioning, natural living, and affective states (FRASER, 2008; MOLENTO, 2012). The basic health and biological functioning sphere seeks to ensure the proper functioning of the body, focused on aspects of health, nutrition, reproduction, and growth (BARNETT et al., 2001). Welfare is the “state of the organism during its attempts to adjust to its environment” (BROOM, 1986). The natural living sphere focuses on the importance of the animal living as if it were in its natural habitat, in this case, the breeding environment must provide conditions for the animals to express their natural behavior as if they were in the wild (DUNCAN; FRASER, 1997). The affective state sphere seeks to ensure positive experiences for animals (comfort, contentment, and positive social interactions) and reduce negative experiences (frustration, fear, pain, and suffering). The focus is on feelings, emotions, and affective states (DUNCAN, 2005).

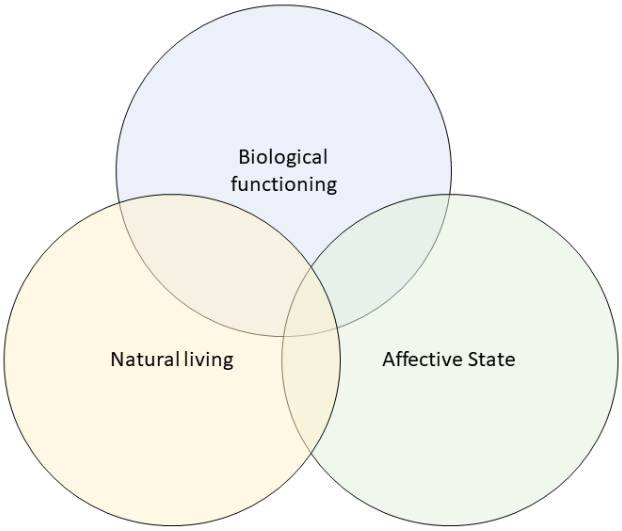

Figure 1. Three animal welfare conceptions. Adapted from Fraser (2008)

For broiler chickens, several factors affect animal welfare, such as husbandry conditions, walking ability, bird health, the expression of natural behaviors through a more complex environment, and positive experiences affecting the affective states throughout rearing (MELUZZI; SIRRI, 2009; JACOBS et al., 2023).

2.2 Husbandry conditions and broiler welfare concerns

The production of chickens on an industrial scale is the most adopted husbandry system in the world poultry chain, as it meets the needs of poultry farmers and guarantees good profitability. Intensive husbandry meets the demands of the national market, stands out in the international market, and meets the farmers’ goals with more return (BASSI; SILVA, 2017). Breeding is usually done in large poultry barns equipped with feeders, drinkers, and bedding material with varying technology levels. It presents a stocking density between 31 and 42 kg/m², varying according to the region weather, the year season, the barn ambiance, and the slaughter age (FAIRCHILD, 2005). Industrial breeding stands out for its high productivity and high investments in genetics, nutrition, health, and management that contribute to the evolution of breeding (ABREU; ABREU, 2011).

Other factors, such as legislation, council recommendations, or farmer associations, influence the stocking density used in poultry barns. In Brazil, the welfare protocol of the Animal Protein Brazilian Association suggests a maximum breeding density of 39 kg/m² (ABPA, 2016). In the United States of America, the National Chicken Council (2017) recommends that the maximum stocking density be related to the slaughter weight, for example, 31.7 kg/m², 36.6 kg/m², 41.5 kg/m², and 43.9 kg/m² related to the slaughter weights of 2.0 kg, between 2.0 kg and 2.5 kg, between 2.5 kg and 3.4 kg, and greater than 3.4 kg live weight, respectively. The European Union allows a maximum of 39 kg/m², which may exceed up to 3 kg/m² if specific criteria are met. In Germany, the maximum density is also 39 kg/m² (LOUTON et al., 2018), while in Norway, the legislation only allows 36 kg/m² (VASDAL et al., 2019).

When breeding on an industrial scale is analyzed, it is observed that there have been significant advances in the animals' comfort through improvements in the ambiance of the poultry houses to meet the requirements of the birds' thermoneutral zone (ABREU; ABREU, 2011). Additionally, there have been advances in sanitary aspects, animal nutrition, and reduction of lameness with genetic improvement (ABREU; ABREU, 2011). However, this husbandry model still receives criticism, and improvements are needed to improve the quality of life of the animals (DUNCAN, 2006; CORNISH; RAUBENHEIMER; MCGREEVY, 2016).

From a practical point of view, environments such as poultry barns lack resources that stimulate the expression of natural behaviors such as foraging, dust bathing, and perching, which restricts animals to environmental and behavioral stimuli (COSTA et al., 2012; TAHAMTANI et al., 2018; LOURENÇO DA SILVA et al., 2021). The husbandry environments have food, water, and shelter easily available through well-distributed feeders and drinkers, in addition to the high stocking density that reduces the willingness to explore and, consequently, makes it impossible for the animal to express its natural behavior, causing a negative impact on the animal's quality of life and leading to higher incidences of lameness (HAYE; SIMONS, 1978; COSTA, 2002; SANS et al., 2014).

2.3 Impacts of lameness on welfare

At the beginning of the 21st century, farms recorded a high lameness prevalence causing economic losses of up to 40% (MENDES et al., 2016). These problems are multifactorial and are related to genetic factors (WEEKS et al., 2000; ALVES et al., 2016a), nutritional imbalance (COOK, 2000; WALDENSTEDT, 2006), husbandry conditions (BIZERAY et al., 2002; CORDEIRO; NÄÄS; SALGADO, 2009), age (BILGILI et al., 2006), stocking density (SORENSEN; SU; KESTIN, 2000), incubation conditions (OVIEDO-RONDÓN et al., 2009) and materials used as bedding (ALMEIDA PAZ et al., 2010). Genetic improvement has managed to reduce to acceptable levels the prevalence of these disturbances in the musculoskeletal system of birds. Currently, birds first develop a bone structure and then gain muscle mass (HARTCHER; LUM, 2020). However, the environment where birds are reared still does not favor increased exploratory activity, reducing locomotor activity, consequently registering lameness and restricting the expression of natural behaviors (LOURENÇO DA SILVA et al., 2021).

Lame birds remain seated on the tibiotarsometatarsal joints most of the time, with a greater predisposition to the development of wounds on the feet, burns in the tarsal region (contact dermatitis), and blisters on the chest that can therefore depreciate the carcass (KESTIN; SU; SØRENSEN, 1999; SANOTRA et al., 2001). Due to the painful experience caused by locomotor disorders, modern broilers spend more time lying down and resting, between 53 to 86%, compared to their ancestor, the red junglefowl, approximately 10% (MURPHY; PRESTON, 1988; DAWKINS, 1989; WEEKS et al., 2000; ALVINO; ARCHER; MENCH, 2009; RIBER, 2015).

Walking ability is the main indicator used by importing countries, which have stricter legislation, to assess animal welfare (CORDEIRO; NÄÄS; SALGADO, 2009). The gait score, initially developed by Kestin et al. (1992), is a practical method that evaluates the gait of birds along a path of one linear meter. Garner et al. (2002) improved the method by removing intermediate scores and assigning scores ranging from 0 to 2, with 0 being a bird that walks normally and takes at least ten uninterrupted steps, 1 - a bird that has difficulty walking and takes between six and ten uninterrupted steps; and 2 - a bird that walks with great difficulty and takes less than six uninterrupted steps or does not walk at all (GARNER et al., 2002).

The main locomotor problems commonly found that affect the gait score and, consequently, broiler welfare are: spine and joint deviations (spondylolisthesis, valgus and varus), tibial dyschondroplasia, femoral degeneration, pododermatitis, and dorsal cranial myopathy (BERNARDI, 2011; ZIMERMANN et al., 2011; AMARAL et al., 2017). Hereinafter, spondylolisthesis stands out in this study, which presents a subclinical phase silently compromising broiler welfare (DINEV, 2013).

Spondylolisthesis, also called kinky back, is a deformity caused by broilers' disturbed growth of the thoracic vertebral arch, with a higher prevalence in the sixth vertebra (T6). It compresses the spinal cord bringing locomotor difficulties and leading to paralysis of the pelvic limbs in severe cases (KELLY, 1971; ABBASABADI; GOLSHAHI; SEIFI, 2021). Commonly, this condition affects birds between the 3rd and 6th week of age before progressing to clinical cases. (DINEV, 2013). Between 15 to 47% of the flock is affected by the subclinical form of spondylolisthesis, while about 2% of the animals show clinical signs such as lameness and the behavior of sitting with the feet outstretched or falling on one side (HOWLETT; WOOD, 1984; JULIAN, 2004; CRESPO; SHIVAPRASAD, 2008; ALVES et al., 2016a).

Previous studies have suggested that the main cause of this condition is a genetic predisposition (OSBALDISTON, 1967; WISE, 1970; KELLY, 1971). According to Alves et al. (2016a), indigenous chickens (slow-growing) have better postural balance conditions, better gait scores, and no prevalence of subclinical spondylolisthesis compared to commercial strains (fast-growing). However, other studies have not confirmed this hypothesis (ALMEIDA PAZ et al., 2010; DINEV, 2012). Likely, a combination of genetic predisposition and environment (rearing and nutrition conditions) may cause the high prevalence of subclinical spondylolisthesis (ABBASABADI; GOLSHAHI; SEIFI, 2021).

Due to the locomotor system immaturity, modern chicken strains have weak tendons and bones to support the body weight caused by rapid muscle growth and exacerbated development of the *Pectoralis major* muscle (ALVES et al., 2016a). This condition changes the center of gravity and posture of the birds, leading to an overload on the locomotor system, which affects walking ability and increases the prevalence of lameness (WEEKS et al., 2000; ALVES et al., 2016b). In another study evaluating the provision of sawdust fiber-diluted diets in the first week of life, Wise (1970) found a lower prevalence of subclinical spondylolisthesis for birds that received high fiber concentrations, but daily gain was also stunted.

Lameness is the main factor affecting broiler welfare, recent research has found evidence that lameness can be minimized due to the positive effects of skeletal and muscular development in broiler chickens with increased locomotor activity (REITER; BESSEI, 2009; RUIZ-FERIA et al., 2014; LOURENÇO DA SILVA et al., 2021; JACOBS et al., 2023). Thus, researchers and industry are developing and adopting new technologies to increase the locomotor activity of animals in the barn and, consequently, reduce lameness prevalence, also known as environmental enrichment (KELLS; DAWKINS; CORTINA BORJA, 2001; BAILIE; BALL; O'CONNELL, 2013; DE JONG; GOËRTZ, 2017).

2.4 Environmental enrichment

Environmental enrichment is a management strategy adopted by industry and researchers to increase the complexity of the environment for animals kept in captivity (BELZ et al., 2003). Modifications are made to the environment to increase behavioral possibilities, reduce abnormal behavior in the species, increase exploratory activity in the environment, and improve biological functions that allow animals to cope with behavioral and physiological challenges throughout their lives (NEWBERRY, 1995; RIBER et al., 2018).

The economic success of implementing environmental enrichment in a production system depends on some criteria, namely: increasing species-specific behaviors, maintaining or improving animal health, maintaining or improving flock productivity, and being practical, easy to implement, and cleaning (VAN DE WEERD; DAY, 2009; RIBER et al., 2018). For broilers, objects such as hay bales, dust bath substrates, perches, pecking objects, and raised platforms are most commonly developed, implemented, and evaluated as environmental enrichment (RIBER et al., 2018). These resources are provided to broilers from different strategies, being used permanently and temporarily throughout each production cycle (JACOBS et al., 2023).

There is a certain level of inconsistency in the literature about the impacts of each environmental enrichment resource on broiler performance, which may vary according to the type of resource and strategy used. For instance, studies evaluating hay bales, peat, perches, platforms, pecking objects (hanging metal chains), dust baths, balls, and mirrors for fast-growing chickens found no effects of these enrichments on final body weight, feed conversion ratio, mortality or percentage of animals rejected at the slaughterhouse (VENTURA; SIEWERDT; ESTEVEZ, 2010; YILDIRIM; TASKIN, 2017; JONG; GUNNINK, 2018; VASDAL et al., 2019). On the other hand, other studies have reported that animals with access to perches and ramps reduced final body weight and feed intake compared to a control group, but no effect on feed conversion ratio was observed (MARTRENCHAR et al., 2000; RUIZ-FERIA et al., 2014). Ohara et al. (2015), assessing hay bales and perches for slow-growing broilers, found better average daily gain and final body weight, but worst feed conversion ratio and mortality compared to the control group. Due to these inconsistencies, more studies are needed to understand environmental enrichment effects on performance.

Despite the inconsistencies about the environmental enrichment impacts on performance, its use for broiler chickens improves behavioral, physiological, health, and cognitive parameters (JACOBS et al., 2023). Previous studies have shown that access to perches, platforms, hay bales, light projectors, and pecking objects increased locomotor and exploratory activity and leg health compared to a control group (TABLANTE; ESTEVEZ; RUSSEK-COHEN, 2003; VENTURA; SIEWERDT; ESTEVEZ, 2010; ZHAO et al., 2013; BAILIE; O'CONNELL, 2015; BERGMANN et al., 2016; KAUKONEN; NORRING; VALROS, 2016; LOURENÇO DA SILVA et al., 2021).

In addition to increased locomotor activity and improved leg health, the use of hay bales, platforms, light projectors, dust baths, vertical panels, mirrors, balls, and pecking objects reduces fear expression and promotes more behavioral opportunities (YILDIRIM; TASKIN, 2017; TAHAMTANI et al., 2018; ANDERSON et al., 2021a; LOURENÇO DA SILVA et al., 2021), besides promoting the development of cognitive functions, including learning and memory (TAHAMTANI et al., 2018).

A complex and dynamic environment promoted by environmental enrichment stimulates positive emotions and behaviors and positively impacts the chickens' affective states (JACOBS et al., 2023). Access to perches, dust baths, light projectors, balls, and pecking objects for broilers reduced negative affective states such as anxiety and pessimism compared to broilers raised in a barren environment (ANDERSON et al., 2021a, 2021b).

2.5 Cognition, emotions, and affective states

Throughout a broiler chicken life, the rearing environment provides a range of information, such as feeders, drinkers, litter, equipment noise, temperature, humidity, and conspecifics and human presence. The chicken then acquires, processes, and stores this information using information-processing mechanisms such as sensory perception and memory, called cognition (PAUL; HARDING; MENDL, 2005; SHETTLEWORTH, 2009; BETHELL, 2015; ROELOFS et al., 2016; MARINO, 2017). From this information collected by cognitive mechanisms, the individual's behavioral response will be influenced by an emotional response and an affective state (HORBACK, 2019).

Emotions are individual short-lived experiences that usually last seconds or minutes. They are object- or event-related and are more intense (KREMER et al., 2020). When a stimulus is presented to the chicken (i.e., a mealworm, an environmental enrichment, a predator presence, high temperature, and a conspecific playing or exploring something), the chicken will present an emotional response to that specific stimulus (PAUL; HARDING; MENDL, 2005). This emotional response is a discrete event that quickly dissipates when the stimulus is removed (JACOBS et al., 2023). According to Paul; Harding; Mendl (2005), emotional response refers to the physiological and neural processes that give animals the ability to avoid harm or punishment and seek valuable resources or rewards.

An animal's emotions include fear, rage, panic, care, seeking, play, and lust (PANKSEPP, 1998). The animal's emotional response to a given stimulus involves subjective, physiological, behavioral, and cognitive components (PAUL; MENDL, 2018), and falls on a two-dimensional spectrum of valence and arousal (HORBACK, 2019). Valence is how the animal perceives a given stimulus, which can be positive (pleasant, rewarding) or negative (unpleasant, punitive) (DE WAAL, 2011; CRUMP et al., 2020). Arousal refers to the intensity of emotion that a given stimulus causes, which can be high or low (DE WAAL, 2011; CRUMP et al., 2020). Emotional response always has a valence level, can vary in arousal and persistence (duration) (PAUL; MENDL, 2018), and can be influenced by long diffuse states called mood or affective states (PAUL; HARDING; MENDL, 2005; MENDL; BURMAN; PAUL, 2010; KREMER et al., 2020).

In contrast to emotion, affective states or moods are individual long-lived experiences that usually last for hours or days. (KREMER et al., 2020). They are not object- or event-related (free floating), are less intense, and can also fall into the valence spectrum (KREMER et al., 2020; JACOBS et al., 2023). For example, an anxious, bored, or pessimistic animal is in a negative affective state. A happy, excited, or optimistic animal is in a positive affective state (JACOBS et al., 2023).

Affective states result from cumulative experience throughout the animal's life (RUSSELL, 2003; BARRETT et al., 2007; KREMER et al., 2020), which means they result from emotional experiences accumulated on scales of valence and arousal over time (MENDL; BURMAN; PAUL, 2010; NETTLE; BATESON, 2012; TRIMMER et al., 2013). As the affective state results from cumulative emotions triggered by the environment, it is also indirectly affected by the environment (KREMER et al., 2020), which can provide us with a big picture of the individual's experience over time (JACOBS et al., 2023). According to Paul; Harding; Mendl (2005), there is ample evidence that both cognitive processes and affective states occur in a causal direction, thus affective states influence cognitive processes and vice versa (LAZARUS, 1982; MENDL et al., 2009; DOLCOS; DENKOVA, 2015). For example, an aspect of the cognitive process such as decision-making can be influenced by the affective state (BECHARA, 2000). In humans and also observed in animals, when the individual is in a negative affective state, it tends to judge an ambiguous stimulus in a negative way (pessimistic), expecting more for an unpleasant experience than for a pleasant, rewarding experience (MACLEOD; BYRNE, 1996; ROELOFS et al., 2016). This situation, when the affective state influences cognitive processes such as judgment, attention, and memory, is defined as cognitive bias. (PAUL; HARDING; MENDL, 2005; MENDL et al., 2009; BETHELL, 2015; FAUSTINO; OLIVEIRA; OLIVEIRA, 2015). Cognitive bias can be used as an indicator of the animals' affective states and can be measured using cognitive bias tests (PAUL; HARDING; MENDL, 2005; MENDL et al., 2009).

Cognitive bias tests are new tools used by ethologists as indicators to measure the affective states of animals and, consequently, welfare. It provides information on how animals perceive their environment (MENDL et al., 2009; WHITTAKER; BARKER, 2020). For instance, the judgment bias test that provides information about the animal's optimism (ROELOFS et al., 2016).

2.6 Judgment bias test

The goal of a judgment bias test is to infer whether the individual is in a positive affective state (optimistic) or is in a negative affective state (pessimistic) (MENDL et al., 2009). An ambiguous cue is presented to the individual, who in turn will use cognitive mechanisms, judge and act as a result of an emotional response biased by the affective state (MENDL et al., 2009; BETHELL, 2015; ROELOFS et al., 2016). Based on the animal's behavioral response, questions about how the animal perceives that ambiguous cue and its expected result (positive or negative) are answered. Thus, it is possible to infer the animal's optimism and pessimism levels (MENDL et al., 2009; BETHELL, 2015; ROELOFS et al., 2016).

Initially developed and applied to humans (ROELOFS et al., 2016), the first judgment bias test used in non-human animals was developed by Harding; Paul; Mendl

(2004). The authors used mice kept in predictable and unpredictable environments. The animals were trained to perform two discrimination tasks between two conditioned stimuli, the first task being to press a lever when hearing a tone indicating a positive event (reward) and the second task being not to press the lever when hearing a tone indicating a negative event (punishment). After the rats met the training criteria, the judgment bias test was performed. The animals were exposed to three ambiguous tones without reinforcement (reward or punishment). As an animal behavioral response, the latency to approach the levers when each ambiguous tone was presented was recorded. Rats kept in the predictable environment were faster at pressing the levers when ambiguous tones were played than rats kept in the unpredictable environment, indicating that they expected more reward in an ambiguous situation and therefore were more optimistic (HARDING; PAUL; MENDL, 2004).

Several judgment bias tests have been developed and applied to more than 22 species of non-human animals (LAGISZ et al., 2020). Different cues were applied in these tests, such as visual (SALMETO et al., 2011), olfactory (BOLEIJ et al., 2012), spatial (KIS et al., 2015), tactile (BRYDGES; HALL, 2017), auditory (MURPHY; NORDQUIST; VAN DER STAAY, 2013), and multimodal (combinations between two or more cues) (BETHELL, 2015; ANDERSON et al., 2021b). Animals are trained to discriminate which cue represents a positive event (reward) and which cue represents a negative, neutral, or less positive event (punishment, unrewarded, or low reward, respectively). For this, the test is designed in one of the three types of discrimination tasks developed in the literature for the judgment bias test, namely: (1) Go/No-Go, (2) active choice with positive reinforcement (Go/ Go +) and (3) active choice with negative reinforcement (Go/Go -) (BETHELL, 2015).

In the judgment bias tests that adopted the Go/No-Go discrimination tasks, animals were trained to associate the positive (+) cue with a reward outcome and to associate the negative (-) or neutral (n) cue with a punishment or unrewarded outcomes, respectively (BETHELL, 2015). This type of discrimination task has already been adopted for several species such as dogs (MENDL et al., 2010; BURMAN et al., 2011; STARLING et al., 2014; KIS et al., 2015), swine (DOUGLAS et al., 2012; SCOLLO et al., 2014), goats (BRIEFER; MCELLIGOTT, 2013), sheep (DESTREZ et al., 2013; VERBEEK et al., 2014; GULDIMANN et al., 2015), cattle (NEAVE et al., 2013; DAROS et al., 2014), horses (BRIEFER FREYMOND et al., 2014), cats (TAMI et al., 2011), primates (BETHELL et al., 2012; GORDON; ROGERS, 2015), rats (BURMAN et al., 2008; RICHTER et al., 2012), fish (LAUBU; LOUÂPRE; DECHAUME-MONCHARMONT, 2019; ROGERS et al., 2020) and chickens (SALMETO et al., 2011; HYMEL; SUFKA, 2012; SEEHUUS et al., 2013; DEAKIN et al., 2016; ZIDAR et al., 2018; ANDERSON et al., 2021b).

In the active choice discrimination task with positive reinforcement (Go/Go +) applied in judgment bias tests, the animals were trained to make an active choice in both cues. In this situation, both cues are positive (+), but one has one high-value reward while the other has a low-value reward (MENDL et al., 2009; BETHELL, 2015). This discriminative task was developed to mitigate a possible lack of motivation behind the 'No-Go' response of the Go/No-Go discrimination task, which could lead to animals not approaching ambiguous cues due to lack of awareness, motivation, confusion, or distraction (MENDL et al., 2009; ROELOFS et al., 2016). However, this discrimination task also receives criticism due to the double reward aspect, which would limit the understanding of judgment biases related to negative experiences in the animal's life. (MENDL et al., 2009). This discrimination task has already been adopted in tests of judgment bias for several species such as swine (MURPHY et al., 2015), brown bears (KEEN et al., 2014), primates (POMERANTZ et al., 2012), rats (BRYDGES et al., 2012; CHABY et al., 2013; PARKER et al., 2014) and chickens (HERNANDEZ et al., 2015).

In the judgment bias tests that adopt the active choice discrimination task with negative reinforcement (Go/Go -), animals are trained to associate the positive (+) cue with a reward and associate the negative cue (-) with the end or to avoid a negative stimulus (punishment) (BETHELL, 2015; ROELOFS et al., 2016). This approach is generally not feasible in studies involving animal welfare, being applied more frequently in studies involving pharmacological manipulation using rats (BETHELL, 2015).

In chickens (*Gallus gallus domesticus*), the judgment bias test has been developed and applied to assess affective states using the Go/Go and Go/No-Go discrimination tasks. The effects of environmental conditions (WICHMAN; KEELING; FORKMAN, 2012; SEEHUUS et al., 2013; ROSS et al., 2019; ANDERSON et al., 2021b), feather pecking genetic selection (PICHOVÁ et al., 2021), corticosterone injections (IYASERE et al., 2017), pharmacological reversal in an anxiety-depression model (HYMEL; SUFKA, 2012), anxiety-depression model induction (SALMETO et al., 2011), temperature manipulation (DEAKIN et al., 2016) and acute stress (HERNANDEZ et al., 2015) were assessed on chickens' affective states. The birds showed a negative affective state, being more pessimistic when subjected to a barren environment (ROSS et al., 2019;

ANDERSON et al., 2021b), when injected with high corticosterone levels (IYASERE et al., 2017), or when induced to be anxious and depressed (SALMETO et al., 2011; HYMEL; SUFKA, 2012). However, some studies have reported unexpected results, for example, high feather pecking strain or acutely stressed chickens being more optimistic. (HERNANDEZ et al., 2015; PICHOVÁ et al., 2021), or a high-complexity environment not inducing a more positive affective state compared to a control group (WICHMAN; KEELING; FORKMAN, 2012; ROSS et al., 2019).

These unexpected results add to a series of limitations found in judgment bias tests, such as time-consuming during training phases (CRUMP; ARNOTT; BETHELL, 2018), which would make application unfeasible under commercial conditions; side bias due to stressful manipulations on the animal's affective states caused by treatments (MENDL et al., 2009), which would reduce the animals' learning ability in discrimination tasks; and learning of ambiguous clues due to the need to repeat the test (DOYLE et al., 2010; WHITTAKER; BARKER, 2020). In chickens, the training phases are long and present low learning success rates (WICHMAN; KEELING; FORKMAN, 2012; SEEHUUS et al., 2013; HERNANDEZ et al., 2015; DEAKIN et al., 2016; IYASERE et al., 2017; ANDERSON et al., 2021b), which impairs the test practicality. This may be associated with how the test is developed and applied to the species, with all training phases and the test phase conducted individually (ROELOFS et al., 2016) for a species that is highly influenced and dependent on social factors for the development of cognitive processes such as learning and memory (NICOL, 2006; MARINO, 2017), which could influence performance during the training and testing phases. According to Marino (2017), chickens have more complex cognitive, emotional, and communicative processes besides social behavior.

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CHAPTER 2

Providing environmental enrichments can reduce subclinical spondylolisthesis prevalence without affecting performance in broiler chickens

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Abstract

Environmental enrichment can increase the occurrence of natural behavior and improve leg health and other animal welfare outcomes in broiler chickens. This study aimed to assess the effects of three environmental enrichments, specifically hay bales, step platforms, and laser lights, on subclinical spondylolisthesis prevalence, productivity, behavior, and gait of broiler chickens (*Gallus gallus domesticus*). Twenty-four hundred day-old male Ross® AP95 chicks from a commercial hatchery were used in a completely randomized design with four treatments and four replicate pens per treatment. Pens contained either a Control (C) treatment, an environment similar to a commercial broiler chicken system without environmental enrichments, or an environment with either additional hay bales (HB), additional step platforms (SP), or additional laser lights (LL). Performance, yield, behavior (frequencies), gait score, and subclinical spondylolisthesis prevalences were assessed. When raised with SP or LL access, fewer chickens had subclinical spondylolisthesis than chickens without enrichments (C) or with HB access. Chickens with access to SP exhibited higher wing yield and less abdominal fat than animals from the C group. Chickens from the LL and HB treatments explored more and rested less frequently than animals from the C and SP treatments. As chickens aged, they became less active, exploring less and increasing resting and comfort behaviors. Treatments did not affect gait. Gait was not associated with subclinical spondylolisthesis prevalence. Environmental enrichments benefitted chicken health (subclinical spondylolisthesis) and behavior (exploration) without negative consequences for performance and yield.

Keywords: Behavior, environmental complexity, leg health, gait score, poultry

Introduction

Locomotory activity is strongly associated with broiler chicken welfare; many behavioral patterns that depend on locomotion, such as exploration, seeking food, water, shelter, and escaping predators, are negatively affected by the poor walking ability in fast-growing broiler chickens [1]. Rapid muscle growth and exacerbated development of the *Pectoralis major* muscle in fast-growing broiler chickens change the chickens' center of gravity, altering the chickens' posture and load on the skeleton compared to slower-growing strains, leading to skeletal-biomechanical imbalances, in turn affecting walking ability and resulting in locomotor disorders [2,3].

Spondylolisthesis, also known as 'kinky back', is a deformity that affects the thoracic vertebral arch of chickens. It occurs in the sixth vertebra (T6), leading to spinal cord compression and locomotor difficulties and, in turn possibly resulting in paralysis of the pelvic limbs in severe cases [4,5]. This condition can often subclinically affect chickens between 3 and 6 weeks of age before it evolves into clinical cases [6]. Subclinical spondylolisthesis can affect 15 to 47% of the flock, while clinical spondylolisthesis can affect 2% of the flock [7–9]. Chickens with subclinical spondylolisthesis do not show symptoms, while broilers with clinical spondylolisthesis are lame and will sit with extended feet, show an imbalance, and fall on their side when attempting to stand [8,10–13]. A positive correlation between gait score and subclinical spondylolisthesis suggests this is a health and animal welfare concern [7].

The main cause of this condition is a genetic predisposition. Indigenous chickens (slow-growing) show a good balance, better gait, and no subclinical spondylolisthesis compared to fast-growing chickens [5,7,11,12]. However, recent studies did not confirm this [14,15]. It is probable that a combination of both genetic predisposition and environment (housing, nutrition) can lead to high prevalences of subclinical spondylolisthesis [4]. According to [7], tendons and bones in recent strains of broiler chickens are too weak to support the chickens' body weight due to the immaturity of the musculoskeletal system. When providing diets diluted with wood sawdust fiber in the first week of life, prevalence of subclinical spondylolisthesis was reduced but daily gain was also stunted [16].

Locomotor disorders can to some extent be prevented by increasing animal activity, as locomotion can positively impact skeletal development [17,18]. Positive activity such as exploratory behavior and locomotion can be stimulated through environmental enrichments, such as straw or hay bales, platforms, and moving laser lights [17–20].

Environmental enrichments increase the complexity of broiler chicken environments and enhance the expression of natural behaviors, i.e., foraging, perching, and playing [19,21]. Foraging is a highly motivated behavior associated with exploration and the appetitive phase of feeding behavior. Chickens peck and scratch the ground searching for food while collecting environmental information [22]. Perching is natural resting behavior; chickens seek an elevated place that allows them to perform surveillance behavior against predators [23,24]. Playing is another natural and social behavior; chickens run, jump, and interact with inanimate objects and conspecifics in a non-aggressive way [25,26].

Potentially beneficial enrichments are straw or hay bales, platforms, and moving laser lights. Hay bales can increase chicken activity by stimulating pecking, foraging, preening, worm running (play), and can provide a barrier for resting [27–29]. Platforms provide animals an elevated area for resting with fewer physical challenges compared to perches [17,23]. In addition, platforms allow chickens to perform natural locomotory behavior, such as jumping and walking up and down a ramp, which may improve chickens' musculoskeletal strength and coordination, and in turn prevent skeletal-biomechanical imbalances [19,30]. Laser light enrichments have been used in previous research and seem a valuable resource to increase broilers' locomotion [19,31–33]. Laser lights can simulate the presence of an insect, stimulating chickens to approach the stimulus [34] and increasing locomotion during early life [19,34], which in turn may benefit the development of their musculoskeletal system.

The effects of environmental enrichments on performance and yield depend on the type of resource used [35]. Studies assessing peat, bales, elevated platforms, perches, mirrors, balls, dust bathing substrates, and pecking objects (hanging metal chains) at 5 or 6 weeks of age for fast-growing broilers have reported no effects of these enrichments on final body weight, feed conversion ratio, mortality, or the percentage of animals rejected in the slaughterhouse [30,36–38]. In contrast, access to perches and ramps reduced body weights and feed intake compared to the control group, with no difference in feed conversion ratios [39,40]. Average daily gain and body weights improved in slow-growing chickens at 9 weeks of age when they had access to bales and perches, but feed conversion ratio and mortality were worsened compared to the control group [41]. Due to these inconsistencies, more research is needed to understand the impact of environmental enrichments on productivity.

Potential effects of environmental enrichments on subclinical spondylolisthesis have not previously been examined. Therefore, this study aimed to assess the effects of three environmental enrichments, specifically hay bales, step platforms, and laser lights, on subclinical spondylolisthesis prevalence, performance, behavior, and gait of broiler chickens. We hypothesized that each environmental enrichment would increase exploratory behaviors in broilers compared to an unenriched control. In turn, we hypothesized that enrichments would reduce the prevalence of subclinical spondylolisthesis (platforms>bales>laser lights) and improve gait scores without affecting productivity compared to an unenriched control.

Materials and methods

The experiment was carried out at the School of Veterinary Medicine and Animal Sciences (FMVZ) at São Paulo State University, Botucatu, SP, Brazil (22° 49’ 07” S and 48° 24’ 40” W). The experimental protocol was approved by the Animal Use Ethics Committee of FMVZ (number 0045/2020 CEUA).

Chickens, facilities, and management

Twenty-four hundred day-old male Ross® AP95 chicks from a commercial hatchery were used. The trial was carried out in a climate-controlled poultry barn featuring negative pressure ventilation. The barn contained sixteen pens (4 x 3 m) provided with 10 cm new wood shavings as bedding, three semi-automatic feeders (one feeder for 50 chickens), and a nipple drinker line (one nipple for 10 chickens). The litter was turned on days 17, 24, and 31. Each pen contained 150 chickens and a targeted maximum stocking density of 39 kg/m² [42]. Pens contained heat lamps in the first 10 days. House temperature was gradually decreased from 32°C on day 1 to 21°C on day 28 and remained 21°C until day 42. The chickens were maintained on an artificial lighting program of 24L:0D in the first 10 days due to the heat lamps and 16L:8D until the end of the trial. The corn- and soybean-meal-based diets were adapted from [43] and met the nutritional requirements in three rearing phases: starter (1-21 days, 24% CP and 3,000 kcal ME/kg), grower (22-35 days, 22.5% CP and 3,150 kcal ME/kg), and finisher (36-42 days, 19% CP and 3,250 kcal ME/kg). Both feed and water were provided *ad libitum*.

Experimental design

The trial followed a completely randomized design with four treatments and four replicate pens per treatment. Pens contained either a Control (C) treatment (Fig. 1A), which consisted of an environment similar to commercial broiler chicken husbandry without environmental enrichments, or an environment with either additional hay bales (HB), step platforms (SP), or laser lights (LL). At 42 days of age, the stocking densities (mean±SE) calculated for each treatment were: C = 36.5±0.9 kg/m^2, HB = 36.0±0.9 kg/m^2, SP = 35.8±1.7 kg/m^2, and LL = 37.6±0.6 kg/m^2.

Environmental enrichment

All resources were introduced on the first day and remained available until day 42. One hay bale (75 x 42 x 30 cm, alfalfa hay) per HB pen (150 chickens) was provided and replaced on day 35. The bale was placed between the drinker line and the barn wall (Fig. 1B). Step platforms (60 × 60 × 7 cm for the lower base and 20 × 20 × 7 cm for the upper base) were made from MDF boards and one was provided in every SP pen (Figure 1C). When the litter was turned, the platforms were scraped to remove excreta. Step platforms were placed between the drinker line and the barn wall. One laser light projector per LL pen was placed at 1.5m height (Mini Stage Lighting XX-027, Spooboola, China). The projector emitted approximately 36 green and red laser lights simultaneously with wavelengths of 532nm (50mW) and 650nm (100mW; Figure 1D). These downwards projected lights moved across an area of approximately 12 m^2 at a slow and steady pace. Projectors were turned on twice a day for 15 minutes, at 09:00 and 15:00 hours.

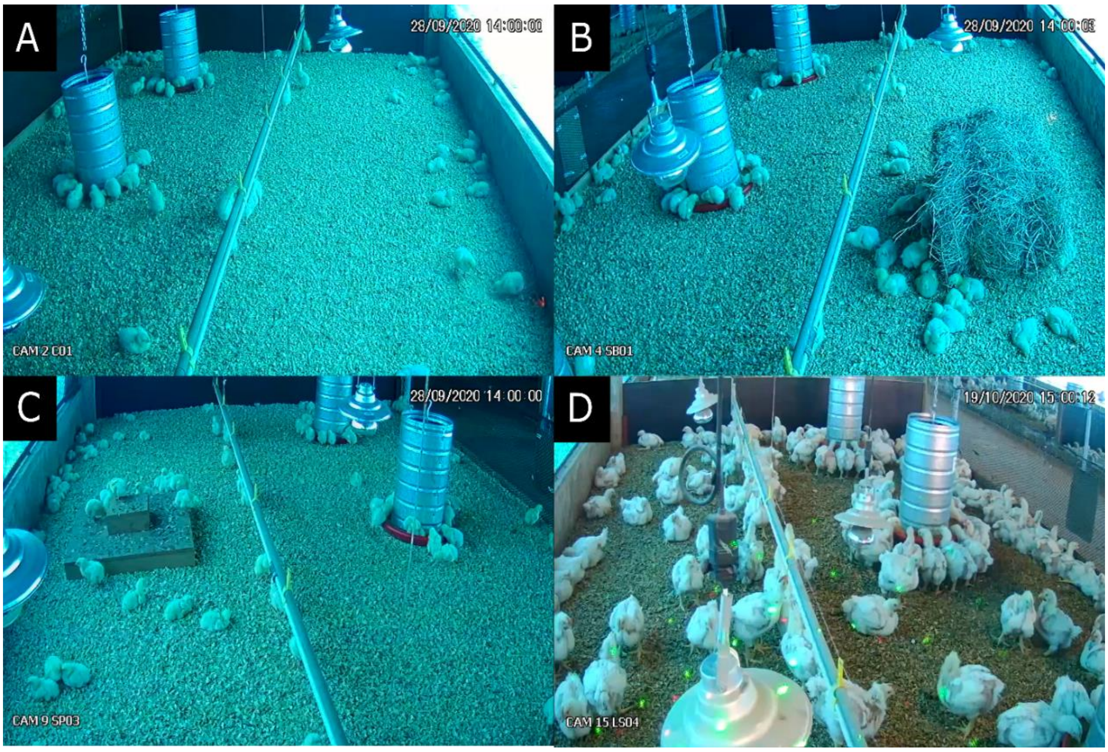

Fig 1. Broiler chickens housed in four environments. (A) Control (B) Hay bale, (C) Step platform, and (D) Laser lights.

Measurements

Behavior

Sixteen high-resolution video cameras (Intelbras, São José, SC, Brazil) were installed at 1.5m height (angled down) to record behavior in each pen (total of 16 pens). Videos were recorded on days 6 (week 1), 13 (week 2), 20 (week 3), 27 (week 4), 34 (week 5), and 41 (week 6). Human disturbance was limited on recording days, and birds were only disturbed for health checks. Frequencies of all selected behavioral occurrences were recorded at pen-level by a single trained observer using 1-minute continuous scan sampling observation with 2 minutes inter-sampling intervals for two 15-minute time periods (starting at 09:00 and 15:00 hours). This resulted in 5 scans per time period and a targeted total of 960 behavioral entries (5 scans × 2 time points × 16 pens × 6 weeks). Behaviors were coded following the ethogram adapted from [44] (Table 1). After recording, behaviors were classified into four categories: consummatory, resting, exploratory, and comfort. Then, the frequencies of each behavioral category were calculated. In addition, the frequency of behaviors associated with the environmental enrichments was assessed individually.

Table 1. Experimental ethogram of recorded broiler chicken behaviors, based on [44].

Behavior	Operational definition
Consummatory	
Eating	Chicken holds its head above the feeding trough or the surrounding area and actively taking in food
Drinking	Chicken is actively taking in water by pecking at nipple drinkers
Resting	
Sitting/resting	Chicken sits on the litter while the head rests on the ground or upright; eyes may be open or closed
Sitting/resting by the bale (HB treatment)	Chicken sits in immediate proximity to bale (within 10 cm) while the head rests on the ground or upright; eyes may be open or closed
Rest on top of bale (HB treatment)	Chicken stands or lies on top of a straw bale
Rest on step platform (SP treatment)	Chicken stands or lies on top of a step platform
Exploratory	
Locomotion	Moving using legs in a continuous forward motion (walking or running), chicken may be flapping wings
Foraging	Pecking and/or scratching at the flooring substrate
Play	After approach of another chicken at high speed, the chicken stops and faces the other briefly, without making physical contact.
Pecking at hay bales (HB treatment)	Chicken uses beak to manipulate hay in the bale
Chasing and/or pecking at light (LL treatment)	Chicken approaches and/or pecks the light emitted by the laser projector
Comfort	
Preening	All behavior patterns associated with cleaning and maintenance of its body surface using the beak; the chicken may stand or lie
Dust bathing	Vertical wing shakes, interacting with flooring substrate, performing side-rubs, and intermittent ground pecking with beak

Gait score

A trained observer assessed all chickens' gait scores (GS) at 21, 28, 35, and 42 days of age. A three-point scale was applied to classify the gait of the chickens according to [45]: score 0 (GS 0) was attributed to healthy chickens that exhibited no abnormality when walking, score 1 (GS 1) was attributed to chickens that exhibited difficulty in walking in a way that was easily identifiable through observation, and score 2 (GS 2) was attributed to chickens exhibiting severe issues walking. Chickens presenting GS 2 were euthanized as a humane endpoint, and their scores were noted. Before gait assessment at 42 days of age, 48 chickens of each treatment (12 per pen) were arbitrarily selected and leg banded for subclinical spondylolisthesis prevalence assessment. Then, gait assessment proceeded in all chickens.

Subclinical spondylolisthesis prevalence

At 43 days of age, the previous leg banded chickens were fasted for 8 hours. Fasting is a common procedure prior to processing to allow emptying of the gastro-intestinal tract and is required by the Brazilian Ministry of Agriculture for food safety considerations (SDA/MAPA Ordinance No. 365, of 16 July 2021). The chickens were weighed and then stunned using an electric stunner (Fluxo UFX 7, Chapecó, SC, Brazil). The chickens were exsanguinated via a cut to the carotid artery and jugular vein. After slaughter, the feet, head, and neck were removed. The chickens' backs were frozen for 48 hours and then sawed sagittally to assess subclinical spondylolisthesis by visualizing the cervical spine between the 6th and the 7th vertebrae macroscopically (Fig. 2). When the vertebrae were found on their normal axis and without compressing the bone marrow, the score was 0 (absence of subclinical spondylolisthesis). When the vertebrae compressed the bone marrow, the score was 1 (presence of subclinical spondylolisthesis) [7,11]. The observer was blinded to the treatments.

Fig 2. Presence or not of subclinical spondylolisthesis in broiler chickens' backs at 43 days of age. (A) Score 0: vertebrae found on their normal axis without compressing the bone marrow, (B) Score 1: vertebrae compressing the bone marrow.

Performance and yield

All chickens and feed were weighed by pen on days 21, 35, and 42 to determine average body weight gain, feed intake, and feed conversion ratio. Mortality and culls were recorded daily. After the slaughter at 43 days, the feet, head, and neck were removed and warm carcasses were weighed to calculate carcass yield (% of live weight). Carcass parts were weighed separately to calculate yields (% of carcass weight) of the breast with skin and bones, wings, legs, back, breast fillet, boneless legs, and abdominal fat.

Statistical analysis

The data were analyzed using SAS Studio 3.8 (SAS Inst. Inc., Cary, NC, USA). The variance homogeneities were assessed by Levene's test and data residuals' normality was verified by the Shapiro-Wilk test. Performance and yield data were subjected to ANOVA using the GLM procedure, followed by Tukey's multiple comparison test, and assigned significance when $P < 0.05$. Behavioral data were subjected to ANOVA using the MIXED procedure, followed by Tukey's multiple comparison test and assigned significance when $P < 0.05$ with treatment ($n = 4$), weeks ($n = 6$), and their interactions ($n = 24$) as fixed effects, pen as a random effect, and time period (9:00 and 15:00 h) as a repeated factor using the "variance components" covariance structure. A generalized linear mixed model was applied for gait score data using a multinomial (ordered) distribution; gait score was the response variable, treatment ($n = 4$), age ($n = 4$), and their interactions ($n = 16$) were fixed effects, and pen was a random effect. Subclinical spondylolisthesis prevalence data were subjected to a generalized linear mixed model using a binary distribution with treatment ($n = 4$) as a fixed effect and pen as a random effect. The correlation between gait score and subclinical spondylolisthesis prevalence was assessed using Spearman's correlation analysis with the CORR procedure, considering a significance of $P < 0.05$.

Results

Behavior

Treatment effects for resting ($F_{3,168} = 3.22$, $P = 0.024$, Fig. 3A) and exploratory ($F_{3,168} = 13.62$, $P < 0.001$, Fig. 3B) behavior frequencies were found. Chickens raised in the control treatment rested more frequently than chickens in the hay bale ($P = 0.018$) or laser light ($P = 0.037$) treatments but at similar frequency compared to chickens in the step platform treatment (P = 0.788). The laser light treatment stimulated more frequent exploratory behavior than hay bale (P = 0.027), step platform (P < 0.001), and control (P < 0.001) treatments. Chickens in the hay bale treatment explored more frequently than those in step platform (P = 0.002) and control (P = 0.002) treatments. Exploratory behavior frequencies for step platform and control treatments did not differ (P = 0.989). No treatment effects were found on comfort ($F_{3,168}$ = 2.16, P = 0.095, Fig. 3C) or consummatory ($F_{3,168}$ = 0.72, P = 0.539, Fig. 3D) behavior frequencies. No interactions between treatment and age were found for any of the four behavioral categories (P > 0.144).

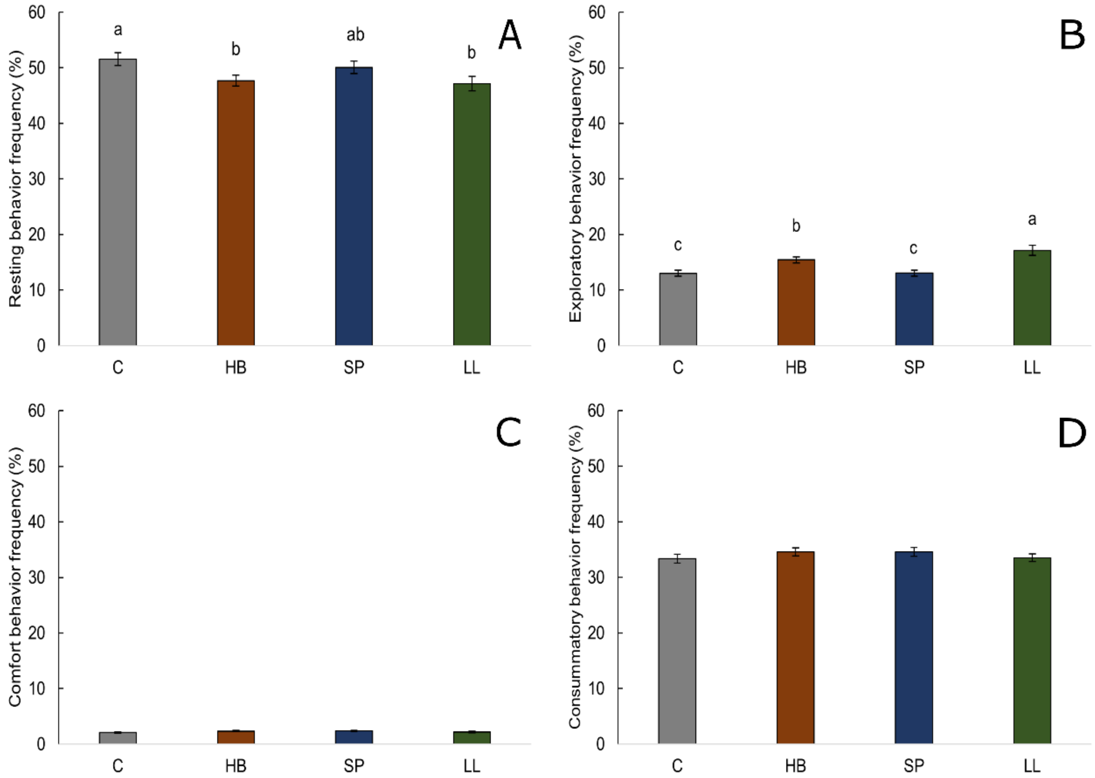

Fig 3. Frequency of observations (mean % ± SEM) of each behavioral category by treatment (C = control, HB = hay bales, SP = step platforms, LL = laser lights) across 2 time periods (09:00 and 15:00 h), n = 192 observations. Frequencies of (A) resting behavior, (B) exploratory behavior, (C) comfort behavior, and (D) consummatory behavior. Means within behavioral category without a common superscript ($^{a–c}$) differed at P < 0.05.

Chickens rested more ($F_{5,168}$ = 2.38, P = 0.041, Fig. 4A) and showed more comfort behavior ($F_{5,164}$ = 34.18, P < 0.001, Fig. 4B) but showed less frequent exploratory behavior ($F_{5,168}$ = 32.62, P < 0.001, Fig. 4C) with age. Similarly, enrichments were used less frequently with age, with decreased frequencies of pecking hay bales ($F_{5,42}$ = 3.56, P = 0.009, Fig. 5), chasing and/or pecking laser lights ($F_{5,42}$ = 10.78, P < 0.001, Fig. 6), and resting near hay bales ($F_{5,42}$ = 5.80, P < 0.001). Increased frequencies of resting on top of hay bales were observed with advancing age ($F_{5,42}$ = 7.13, P < 0.001). Consummatory behavior ($F_{5,168}$ = 0.57, P = 0.719, Fig. 4D) and use of step platforms ($F_{5,42}$ = 1.53, P = 0.201) were not affected by age.

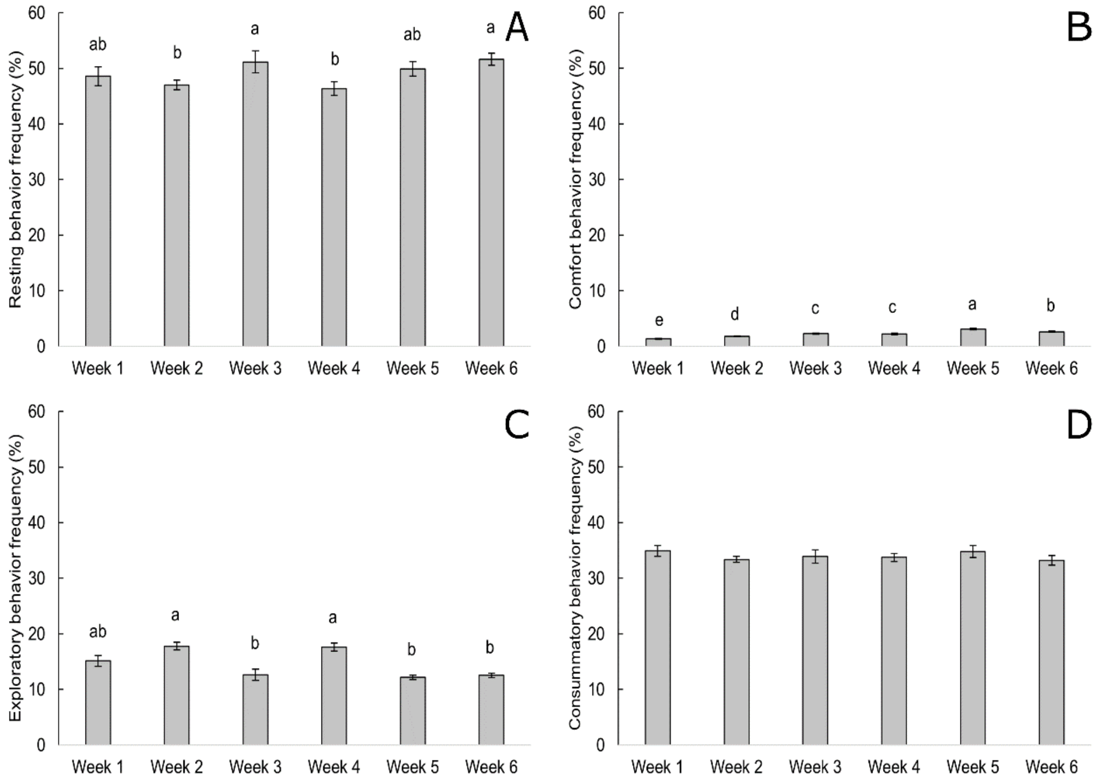

Fig 4. Frequency of observations (mean % ± SEM) of each behavioral category by chicken age (in weeks) across two time periods (09:00 and 15:00 h), n = 192 observations. Frequencies of (A) resting behavior, (B) comfort behavior, (C) exploratory behavior, and (D) consummatory behavior. Means within a behavioral category without a common superscript ($^{a–e}$) differed at P < 0.05.

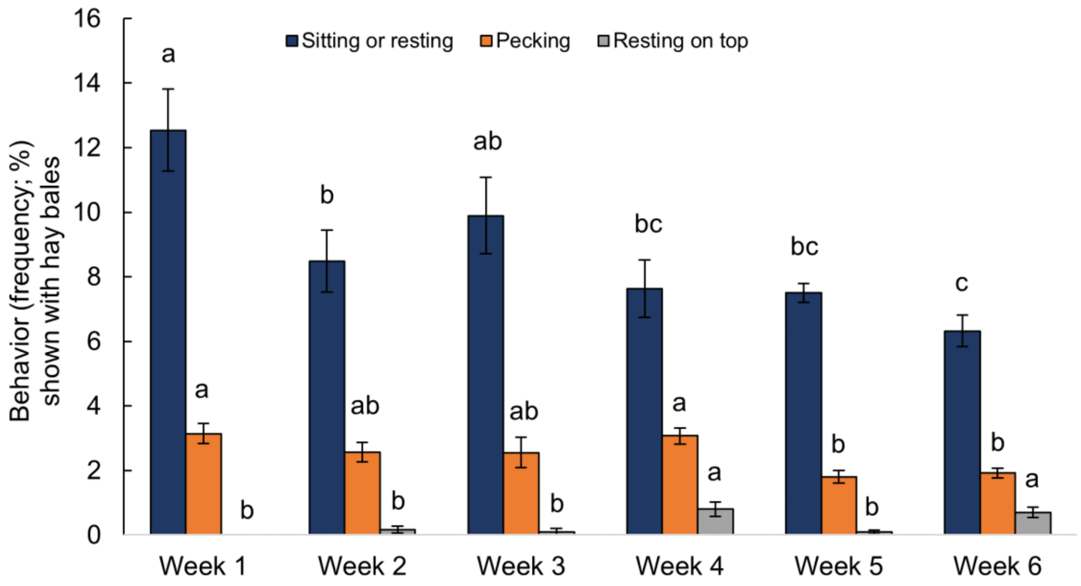

Fig 5. Frequency of observations (mean % ± SEM) of behaviors associated with the provided hay bale by age (in weeks) across two time periods (09:00 and 15:00 h). Sitting or resting by bale (n = 24), Pecking (n = 24), and Resting on top (n = 24). Frequencies without a common superscript ($^{a\text{-}c}$) differed at P < 0.05.

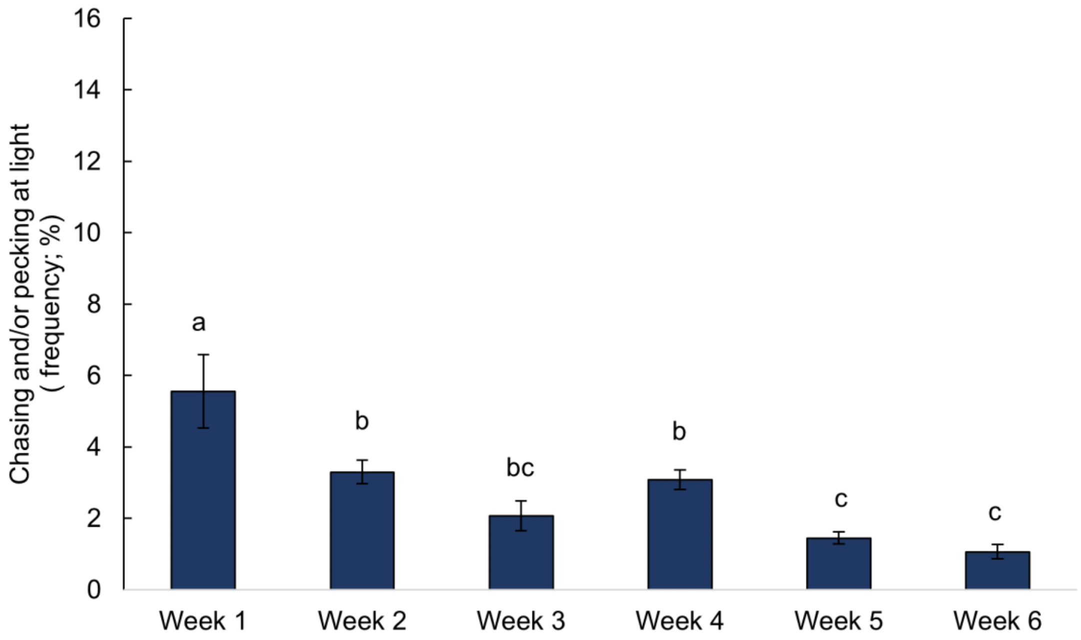

Fig 6. Frequency of observations (mean % ± SEM) of behaviors associated with the provided laser lights by age (in weeks) across two time periods (09:00 and 15:00 h), n = 24. Frequencies without a common superscript ($^{a\text{-}c}$) differed at P < 0.05.

Gait score

Gait scores were affected by age ($F_{3,135} = 37.98$, $P < 0.001$, Fig. 7). Chickens showed better gait at 21 days than at 28 ($P = 0.004$), 35 ($P < 0.001$) and 42 days ($P < 0.001$), at 28 days than at 35 ($P = 0.002$) and 42 days ($P < 0.001$), and at 35 days than at 42 days of age ($P < 0.001$). No interaction between treatment and age was found ($F_{9,135} = 0.24$, $P = 0.989$). Gait was not affected by treatments ($F_{3,135} = 1.37$, $P = 0.251$). Gait was not associated with subclinical spondylolisthesis prevalence ($r = 0.010$, $P = 0.888$).

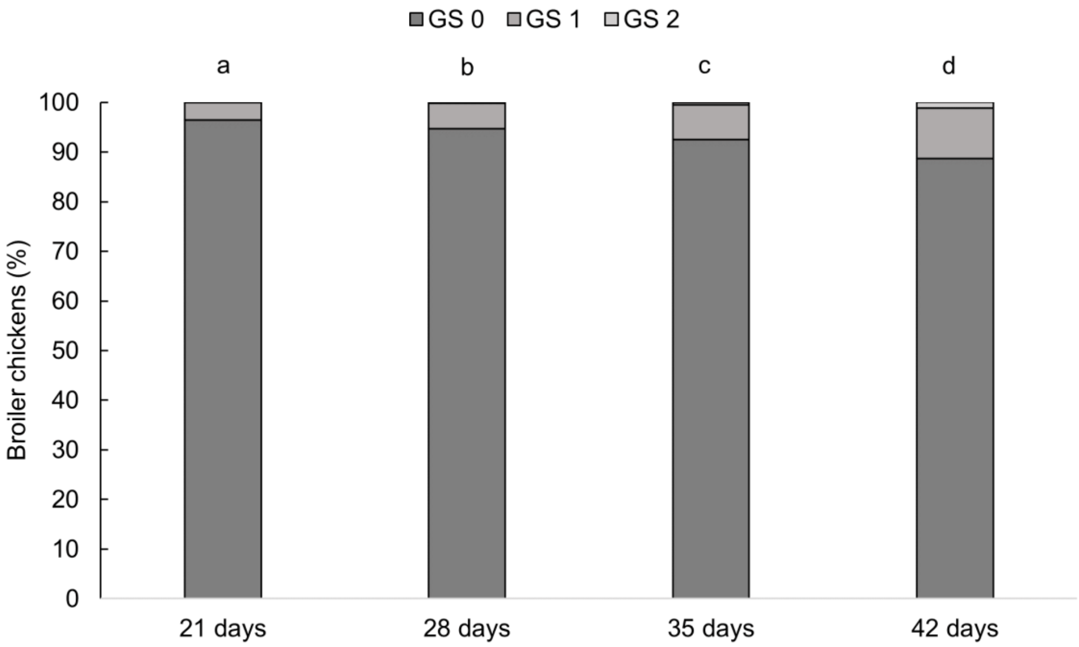

Fig 7. Frequencies (%) of broiler chickens by gait score (GS) at 21, 28, 35, and 42 days of age, n = 16. GS 0: Healthy chickens that exhibited no abnormality when walking, GS 1: Chickens that exhibited difficulty walking in a way that was easily identifiable through observation, and GS 2: Chickens exhibiting severe issues walking [45]. Ages without an uncommon superscript ($^{a–d}$) differed at $P < 0.05$.

Subclinical spondylolisthesis prevalence

Subclinical spondylolisthesis prevalence was affected by treatments ($F_{3,45} = 5.16$, $P = 0.002$, Fig 8). Chickens from the control treatment showed a higher prevalence than chickens from the laser light ($P = 0.044$) and step platform ($P = 0.004$) treatments. Chickens from the hay bale treatment had a higher prevalence than chickens from the step platform treatment ($P = 0.035$). Subclinical spondylolisthesis prevalences did not differ for control and hay bale (P = 0.847), hay bale and laser light (P = 0.245), and laser light and step platform (P = 0.784) treatments.

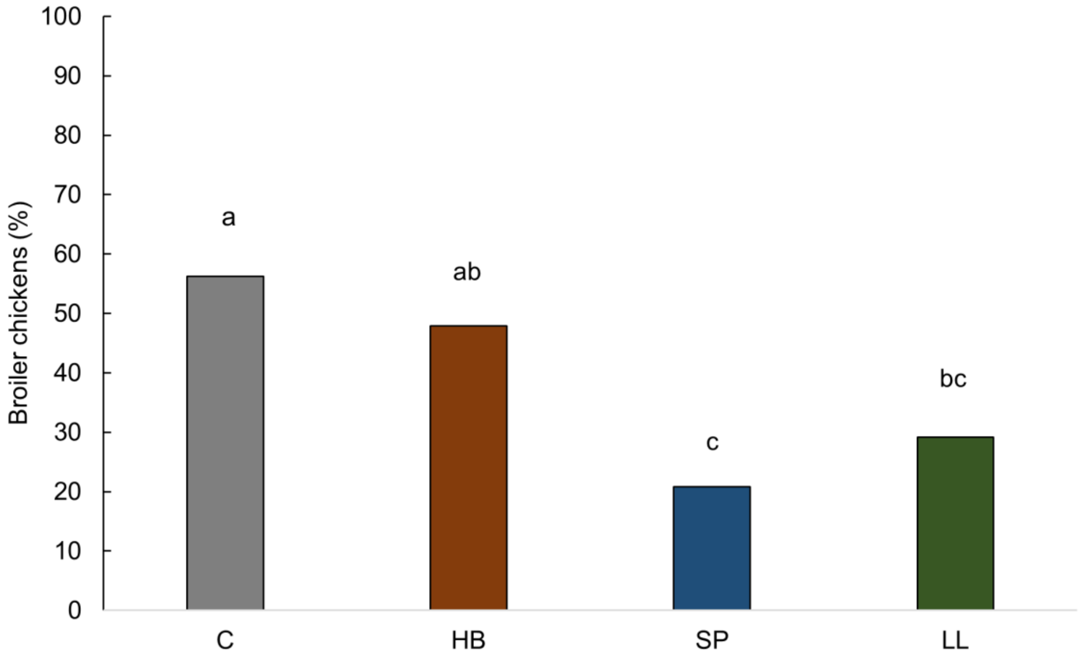

Fig 8. Proportion (%) of broiler chickens with subclinical spondylolisthesis score 1 (vertebrae compressed the bone marrow) at 43 days of age (n = 192). C = control, HB = hay bales, SP = step platforms, LL = laser lights. Bars without a common superscript ($^{a–c}$) differed at P < 0.05.

Performance and yield

Live performance parameters were not impacted by treatments in any of the three rearing phases (P > 0.120, Table 2). Chickens raised with access to step platforms had higher wing yield (P = 0.038) and lower abdominal fat yield (P = 0.048) than chickens raised in the control group (Table 3). Chickens from the laser light treatment had a lower back yield than the control treatment (P = 0.031). No other differences in yield were found (P > 0.107).

Table 2. Performance (mean ± SEM) of broiler chickens between days 1-21, days 1-35, and days 1-42 of rearing, n = 16.

Performance	Treatments[1]				SEM	ANOVA
	C	HB	SP	LL		
1-21 days						
Feed intake (g/chicken/period)	1,188	1,199	1,216	1,211	7.40	$F_{3,15} = 0.68$, $P = 0.583$
Weight gain (g/chicken/period)	920	932	951	951	7.52	$F_{3,15} = 1.04$, $P = 0.412$
Feed conversion ratio	1.29	1.29	1.28	1.27	0.01	$F_{3,15} = 0.52$, $P = 0.680$
Mortality (%)	1.34	2.18	1.01	1.34	0.19	$F_{3,15} = 2.28$, $P = 0.132$
Culls (%)	1.01	0.67	0.67	0.67	0.17	$F_{3,15} = 0.20$, $P = 0.894$
1-35 days						
Feed intake (g/chicken/period)	3,391	3,377	3,425	3,421	21.74	$F_{3,15} = 0.25$, $P = 0.860$
Weight gain (g/chicken/period)	2,201	2,179	2,180	2,251	27.86	$F_{3,15} = 0.32$, $P = 0.811$
Feed conversion ratio	1.54	1.55	1.57	1.52	0.01	$F_{3,15} = 0.73$, $P = 0.556$
Mortality (%)	1.34	2.68	1.51	1.34	0.24	$F_{3,15} = 2.39$, $P = 0.120$
Culls (%)	1.17	0.84	1.51	1.34	0.18	$F_{3,15} = 0.61$, $P = 0.619$
1-42 days						
Feed intake (g/chicken/period)	5,012	4,939	5,013	5,087	42.03	$F_{3,15} = 0.46$, $P = 0.715$
Weight gain (g/chicken/period)	2,999	2,967	2,949	3,093	42.04	$F_{3,15} = 0.53$, $P = 0.669$
Feed conversion ratio	1.67	1.67	1.70	1.64	0.01	$F_{3,15} = 0.71$, $P = 0.562$
Mortality (%)	2.18	3.35	2.18	2.18	0.26	$F_{3,15} = 1.30$, $P = 0.319$
Culls (%)	2.52	2.01	2.52	2.85	0.28	$F_{3,15} = 0.33$, $P = 0.801$

[1] C = Control, HB = hay bales, SP = step platforms, LL = laser lights.

Table 3. Yield (mean % ± SEM) of carcass, breast, wings, legs, back, breast fillet, boneless legs, and abdominal fat of broiler chickens slaughtered at day 43 of age, n = 16.

Yield (%)	Treatments[1]				SEM	ANOVA
	C	HB	SP	LL		
Carcass[2]	75.79	75.93	75.92	76.13	0.14	$F_{3,15} = 0.27$, $P = 0.850$
Breast[3]	40.78	40.82	41.03	41.69	0.15	$F_{3,15} = 1.96$, $P = 0.121$
Wings[3]	10.01 ^{b}	10.12 ^{ab}	10.28 ^{a}	10.08 ^{ab}	0.04	$F_{3,15} = 4.41$, $P = 0.038$
Legs[3]	30.69	31.08	31.14	30.73	0.10	$F_{3,15} = 1.24$, $P = 0.295$
Back[3]	17.46 ^{a}	17.29 ^{ab}	17.06 ^{ab}	16.73 ^{b}	0.09	$F_{3,15} = 3.02$, $P = 0.031$
Breast fillet[3]	31.87	31.97	32.71	32.63	0.15	$F_{3,15} = 2.06$, $P = 0.107$
Boneless legs[3]	24.70	24.89	25.29	24.84	0.11	$F_{3,15} = 1.32$, $P = 0.270$
Abdominal fat[3]	1.43 ^{a}	1.35 ^{ab}	1.29 ^{b}	1.37 ^{ab}	0.03	$F_{3,15} = 3.99$, $P = 0.048$

[1]C = Control, HB = Hay bales, SP = Step platforms, LL = laser lights. [2]Percentage of carcass yield was calculated based on live weight. [3]Part yields were calculated based on carcass weight. Row means without a common superscript ($^{a\text{-}b}$) differed at $P < 0.05$.

Discussion

This is the first study to assess the association between environmental enrichments and subclinical spondylolisthesis prevalences. Here we assessed three enrichments that allow broiler chickens to perform natural behavior, reduce subclinical spondylolisthesis prevalence, and maintain performance and yield. The current study showed that access to step platforms and laser lights reduced subclinical spondylolisthesis prevalence compared to a control, which partially aligns with our hypothesis. The prevalence of subclinical spondylolisthesis in fast-growing broiler chickens was previously associated with their fast breast muscle growth, causing unstable equilibrium and in turn leading to postural distress and pressure on the locomotor system [2,7,15,46,47]. We argue that adding resources to the chickens' environments increases exploratory behaviors and, consequently, locomotion and exercise, which can alleviate pressure on the locomotor system and reduce the prevalence of subclinical spondylolisthesis.

The impact of step platforms and laser lights can be explained by the daily and regular use of step platforms, providing means for exercise, such as walking up, down, and jumping off the platforms, and the increase in exploratory behavior in laser light treatment, such as chasing and/or pecking at the light. This daily exercise likely strengthened their locomotor system, improving their musculoskeletal development [17,18], thus preventing the skeletal-biomechanical imbalance caused by exacerbated growth of the breast muscle [2,3,19]. In line with current results, access to platforms and laser lights increased locomotion and improved leg health in fast-growing broilers [19,20,30,41].

Chickens chased and pecked laser lights more frequently between 1-4 weeks of age compared to older ages. Additionally, chickens used step platforms constantly during their life. This exercise may have stimulated and strengthened their locomotor system at a key timepoint of locomotor system development around 3-4 weeks of age, which is indicated by the body's peak mineral [48] and protein deposition [49] and leg growth rates [50] between 21 and 28 days of age. Thus, exercise especially at that age may have alleviated the impacts of rapid body weight gain and may have reduced postural distress, and in turn contributed to healthy vertebral bone development, reducing the prevalence of subclinical spondylolisthesis.

Access to hay bales did not impact subclinical spondylolisthesis prevalence compared to the control group. The lack of impact could be due to the type of behavior that hay bales stimulated compared to laser lights and step platform enrichments. Chickens with access to hay bales performed more pecking than chickens raised in a barren environment [30,51], a behavior less intensive to the locomotor system compared to for instance jumping, as pecking can be performed while sitting. In addition, access to hay bales reduced locomotion compared to a barren control [51] or did not impact foraging, running, and walking [18,52]. In this study, all these behaviors were grouped in the same category as exploratory behavior, so the effect of treatments on distinct exploratory behaviors was not tested, as this was not the study's objective. Chickens with access to hay bales explored more compared to chickens in the control and step platform treatments. The hay itself was used as a foraging substrate that was not available when housed with wooden step platforms or without enrichments. Previous studies also showed more exploration with access to hay bales compared to a control group [19,20,41,51]. Additionally, our results showed that chickens rarely used hay bales for perching, possibly because it was difficult to access the top of hay bales. Thus, the low occurrence of intense exercise resulted in the lack of impact on subclinical spondylolisthesis prevalence.

Chickens with access to laser lights exhibited more frequent exploratory behavior than chickens in other treatments. Laser lights can simulate insects that elicit foraging and pecking, which are highly motivated natural behaviors also performed by domestic chicken ancestors [32,53]. Similar to our findings, laser lights alone or combined with other enrichments increased broiler chicken activity compared to a control group [19,32,33]. No treatment effect was found on comfort behavior frequencies in the current study, suggesting that enrichments did not stimulate comfort behavior, while substrates such as sand do [27].

As chickens aged, they became less active, interacted with laser lights and hay bale enrichments less, and performed more comfort behaviors. Similar behavioral frequencies were reported in previous work, where fast-growing broiler chickens reduced activity, reduced exploration, and increased comfort behaviors such as preening [19,51,52,54] and dustbathing [19,51,52], likely due to high body weights and relatively immature locomotor systems [19,51,52,54]. Bergmann et al. [44] reported lower preening and dustbathing frequencies than this study, likely due to methodological differences. They observed chickens from 01:00 to 23:00 h (rather than only during light hours), which included long periods of inactivity (during dark hours). We observed behaviors during high-activity hours only, which probably explains the higher frequencies of these comfort behaviors compared to [44]. No treatment and age interaction was found, indicating that the enrichments used in this study did not mitigate the effects of age and weight on behavior.

Performance parameters (body weight, feed intake, and feed conversion ratio) were not affected by the treatments. These results align with other studies that reported that environmental enrichments rarely alter performance [30,36–38]. This suggests that energy requirements for increased activity levels associated with enrichments were negligible. In contrast, environments enriched with platforms, barrier perches, dustbathing areas, and wooden ramps reduced chickens` body weight gain and increased feed intake, which was theorized to be due to increased activity [55]. Similarly, other studies showed that more activity stimulated by outdoor access increased energy requirements and reduced body weight and feed efficiency [56,57]. Yield was impacted by step platform access, with higher wing yield and lower abdominal fat in the step platform treatment than in the control treatment, but no differences between other treatments. We theorize that increased exercise throughout the rearing phase positively impacted wing yield in step platform treatment, as activities such as walking up, down, and jumping off the platforms are often accompanied by vigorous wing flapping [30], which could have increased wing muscle development and reduced abdominal fat.

Exercise increases the expression of a muscle development gene (*MUSTN1*) that is part of a multi-protein transcriptional complex responsible for skeletal muscle hypertrophy regulation [58,59]. In addition, exercise increases serum creatine kinase concentrations, which are positively associated with muscle growth rate [60,61]. Thus, chickens in the step platform treatment may have had increased expression of *MUSTN1* and increased circulating creatine kinase concentrations compared to chickens from control treatment due to exercise, thus more wing muscle development and less abdominal fat.

The increased activity and leg exercise in laser lights and step platform treatments compared with the control group were not reflected in the chickens' gait scores. In addition, the subclinical spondylolisthesis prevalence in this study was not associated with gait scores, which is in line with expectations of a subclinical rather than a clinical diagnosis [8]. Generally, gait scores in the current study were low, reflecting good gait. Gait score is positively correlated with a range of locomotor disorders in broiler chickens, such as spondylolisthesis, tibial dyschondroplasia, valgus angular deformity, and pododermatitis [7]. The development of locomotor disorders is multifactorial, with nutritional imbalances [62–64], environmental conditions [65–67], age and stocking density [68], incubation conditions [69], and bedding material [15] impacting gait. As husbandry conditions in the current study were consistent across treatments and were meeting or exceeding commercial standards, all gait scores were better compared to broilers housed in commercial conditions [70,71]. Thus, the prevalence of lameness was relatively low in the current study, allowing little room for improvement possible by adding enrichments. Previous studies have reported benefits of environmental enrichments on gait [18,72,73]. In this current study, gait worsened with age, probably due to weight gain, as fast-growing broiler chickens are prone to impaired gait as they reach slaughter weight and age [68,70,74].

In conclusion, these data showed that especially access to step platforms, but also to laser lights, reduced subclinical spondylolisthesis prevalence and maintained performance and yield in fast-growing broiler chickens. Furthermore, laser lights and hay bales increased exploratory behavior compared to the control group without enrichments. Gait was good, not impacted under the current study conditions, and was not associated with subclinical spondylolisthesis prevalence. Thus, our results suggest that access to environmental enrichments improved animal welfare by reducing subclinical spondylolisthesis prevalence (step platforms and laser lights) and increasing the frequencies of natural behaviors (hay bales and laser lights) without negatively impacting performance and yield. However, it is important to underline that subclinical spondylolisthesis is a multifactorial locomotor disorder and that many factors, including genetic predisposition, nutrition, environment, age, stocking density, incubation conditions, and bedding material can contribute to the etiology of spondylolisthesis [7,11,13–16]. Our findings suggest that environmental enrichment (step platforms and laser lights) can be used as a tool to reduce subclinical spondylolisthesis prevalence compared to a barren environment (control). These enrichments could be easily applied in commercial practice.

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CHAPTER 3

Social-pair judgment bias testing in slow-growing broiler chickens raised in low- or high-complexity environments

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Abstract

Impacts of environmental complexity on affective states in slow-growing broiler chickens (*Gallus gallus domesticus*) are unknown. Chickens' performance in judgment bias tests (JBT) can be limited as they are tested individually, causing fear and anxiety. The objectives were to apply a social-pair JBT to assess the effect of environmental complexity on slow-growing broiler chickens` affective states, and assess the impact of fearfulness, anxiety, and chronic stress on JBT performance. Six-hundred Hubbard Redbro broilers were housed in six low-complexity (similar to commercial) or six high-complexity (permanent and temporary enrichments) pens. Twelve chicken pairs were trained (1 pair/pen, n = 24 chickens) using a multimodal approach (visual and spatial cues), with reward and neutral cues of opposing color and location. Three ambiguous cues were tested: near-positive, middle, and near-neutral cues. Approach and pecking behavior were recorded. Eighty-three percent of chickens (20/24) were successfully trained in 13 days. Fearfulness, anxiety, and chronic stress did not impact chickens' performance. Chickens successfully discriminated between cues. Low-complexity chickens approached the middle cue faster than high-complexity chickens, indicating that they were in a more positive affective state. The environmental complexity provided in this study did not improve affective states in slow-growing broiler chickens compared to a control. A social-pair JBT resulted in excellent learning and testing outcomes in slow-growing broilers.

Introduction

Positive affective states in chickens can been assessed through the use of cognitive bias tests[1,2]. This sort of assessment can provide insights in affect-induced judgment, attention, or memory bias[3]. Judgment bias testing (JBT) is used to determine levels of optimism and pessimism of individuals based on their responses to ambiguous cues during testing[1]. Generally, this test shows good validity as determined through corroborating methods of affective state assessment. However, JBT findings can be counter-intuitive and exhibit high levels of individual variability[2].

The JBT has been applied to study chickens' affective states using different testing designs and conditions, such as Go/Go and Go/No-Go tasks to assess the effects of environmental conditions[4–7], feather pecking genetic selection[8], impact of corticosterone injections[9], pharmacological reversal and an anxiety-depression model[10,11], temperature manipulation[12], and acute stress[13]. Chickens showed sensibility to distinct conditions and presented more 'pessimistic' judgments when raised in a non-stimulating environment[4,5], when injected with high corticosterone levels[9], or when pharmaceutically induced to be anxious and depressed[10,11,13]. Unexpectedly, high feather pecking lines and acutely stressed hens approached ambiguous cues faster, suggesting optimism associated with these negative states[8,13]. In other studies, a complex environment did not induce a more positive affective state[4,7]. These unexpected outcomes could be associated with testing methodology, as chickens, a social species, were trained and tested individually[4–9,12,13].

The gregarious nature of chickens could potentially be utilized when training and performing the JBT. Social interaction and environment can shape cognition and vice versa[14]. Chickens are influenced by social factors that mediate learning and memory processes[15]. For instance, imprinting behavior and social facilitation stimulate learning[15–18]. Similarly, social isolation during early development provoked distress and hampered spatial learning in adult chickens[19], and social isolation for five or sixty minutes resulted in pessimistic judgments in a JBT[10,11]. In addition, chronic distress can negatively impact cognitive ability associated with learning by shaping neuronal dendritic morphology and decreasing dendritic complexity within the hippocampus[20]. For instance, chronically distressed rodents showed behaviors associated with anhedonia and decreased motivation, impairing their spatial acquisition in appetitively motivated tasks[21–24] similar to a JBT. Thus, a social training and testing approach could potentially improve learning and performance directly through social facilitation, and indirectly through reduced distress.

Individual preferences, fearfulness, and anxiety can impact chickens' learning processes and outcomes in a JBT[25,26]. Prior studies trained and tested birds individually, and training success varied[5–7,9,12,13]. A social approach, where learning is stimulated, could improve training and testing outcomes. Laying hens with a reactive behavioral response learned JBT tasks quicker during training, while fearful and stress-sensitive hens developed side biases and did not meet the JBT training criteria[26]. This suggests that these behavioral traits should be accounted for when performing a JBT study. Social experiences and intrinsic state, i.e., food competition, hierarchy, environmental conditions, individual levels of fat reserves, metabolic rate, and hormone levels shape behavioral responses and individual preferences[27]. This further suggests that individual characteristics (fearfulness, anxiety, and social experiences) should be considered when performing cognitive bias tests.

A monotonous environment common in conventional fast-growing broiler chicken houses does not favor the expression of natural behaviors, i.e., foraging, dustbathing, and perching, which can improve animal welfare and cognition[5,28–30]. Providing enrichments increases the environmental complexity and the expression of these natural behaviors in fast-growing broiler chickens[31–33], and improves aspects of affective state[5]. A similar response may be observed in slower growing broiler chicken strains.

The effects of environmental enrichment on the affective states of slow-growing broiler chickens, and the potential benefit of a social JBT approach have not been examined. Therefore, this study aimed to assess the affective states of slow-growing broiler chickens raised in high- or low-complexity environments, using a novel social-pair JBT approach. We also aimed to evaluate the effects of fearfulness, anxiety, and chronic stress (feather corticosterone) on training and testing performance. We hypothesized that chickens from high-complexity environments would make more optimistic choices than chickens from low-complexity environments. Additionally, we hypothesized that a social-pair approach would attenuate fearfulness, anxiety, and chronic stress effects, resulting in improved learning and thus judgment bias training performance. Improved learning would be reflected in fewer training rounds needed to meet the training criteria.

Methods

Ethics declarations

The trial was carried out at Virginia Tech's Turkey Research Center from March through May 2022. Virginia Tech's Institutional Animal Care and Use Committee approved the experimental protocol as part of a larger experiment (protocol number 21-221). The experiment was performed following relevant guidelines and regulations. This study is reported in accordance with ARRIVE guidelines[34].

Animals and housing

Six hundred day-old male Hubbard Redbro (slow-growing strain) broiler chicks from a commercial hatchery where they were vaccinated for Marek's disease, followed by 6-hour transportation to the research facility. The trial was carried out in a fully automated climate-controlled poultry house with negative pressure ventilation. Chicks were randomly allocated to 12 pens of 8.75 m^2 each, with 50 chicks per pen. Calculated stocking densities at 22, 44, and 53 days of age were 3.88±0.06, 12.14±0.28, and 16.12±0.66 kg/m^2, respectively. All pens contained pine shavings as bedding (at approximately 6 cm depth), two galvanized tube feeders, and two water lines with nipple drinkers. Both feed and water were provided *ad libitum*. The corn-soy diets were prepared according to the nutritional specifications for conventional broiler chickens[35] and were separated into three rearing phases: starter (day 1–22; 3000 kcal ME and 23% CP), grower (day 22–44; 3100 kcal ME and 21.5% CP), and finisher (day 44–53; 3150 kcal ME and 20% CP). House temperature started at 35 °C on day 1, and was gradually reduced to 21 °C on day 29, and remained at 21 ºC until the end of the trial. The chickens were maintained on an artificial lighting program of 24L:0D in the first 4 days due to heat lamps, 20L:4D from day 5 to 7 of age, and 18L:6D until the end of the trial, with a light intensity of approximately 15 lux during light hours.

Experimental design

The trial consisted of a randomized block design of two environmental complexity treatments with six replicates each. The low-complexity control environment provided chickens conditions similar to commercial standards. The high-complexity environment provided chickens permanent and temporary enrichments. Permanent enrichments included a dust bath constructed from lumber (108 cm L x 91 cm W x 10 cm H) filled with playground sand (Quikrete, GA, USA) and two wooden platforms (488 cm L x 45.5 cm W x 7.5 cm H) in each pen, providing 19.5 cm linear perch space per bird (Fig 1). Six temporary enrichments were rotated every three days, with two enrichments in each pen at one time. These enrichments included two plastic treat balls (Ethical Products, Inc., NJ, USA) filled with oats placed onto the litter paired with two bundles of string hung from the pen barrier. Half a cabbage hung at chicken height paired with alfalfa hay provided in two metal cage balls (20.3 cm diameter; Darice, OH, USA) placed on the litter. Two rectangular hanging mirrors (19 x 28 cm) paired with a handful of chicken scratch thrown into the litter (corn, wheat, milo, barley, oat, sunflower seed, and mullet; Manna Pro Products, MO, USA).

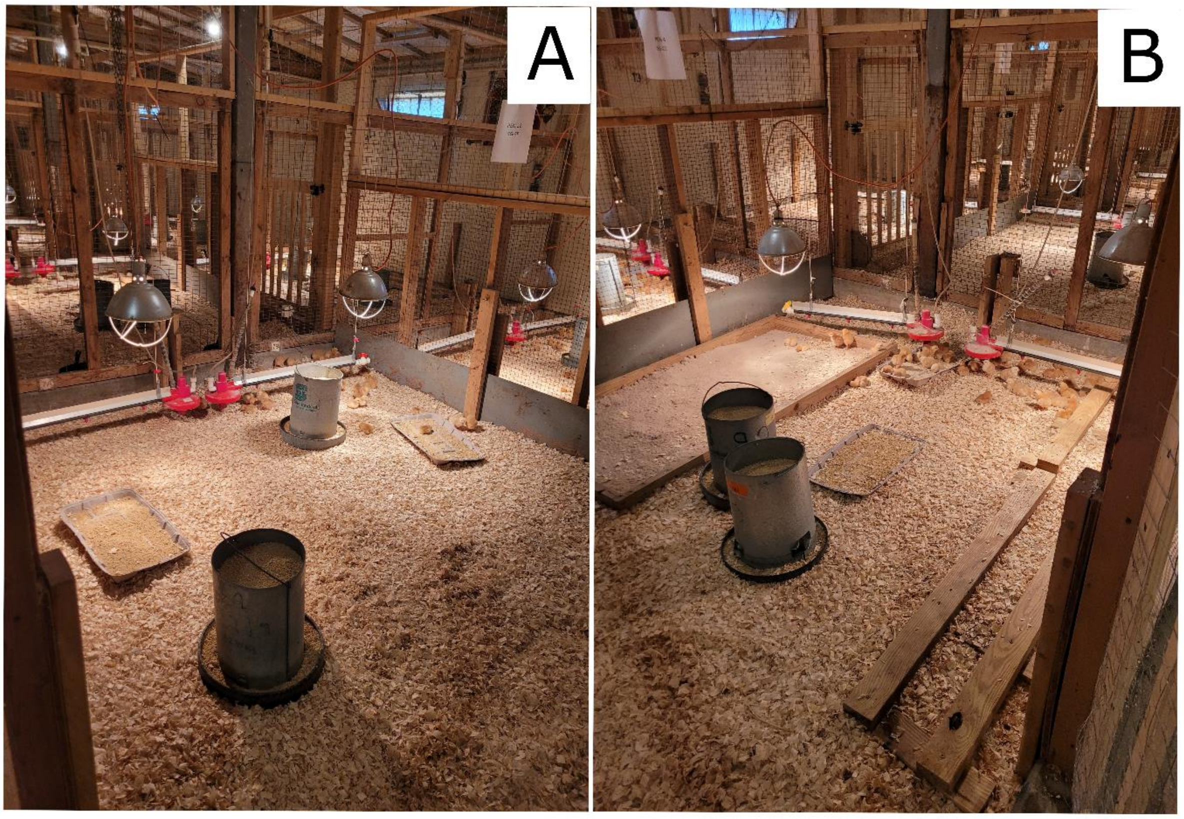

Figure 1. Chickens housed in two complexity environments. (A) Low-complexity, similar to commercial standards with feed, water and shaving; and (B) High-complexity, with permanent and temporary enrichments.

Judgment bias procedure

At 24 days of age, 24 wing-banded chickens (n = 2/pen) were arbitrarily selected and gently marked on wings and legs with a livestock marker (All-Weather Paintstik, LA-CO Industries, Inc., IL, USA). These markings were reapplied as necessary throughout the experiment. The judgment bias procedure followed a 4-step process, including habituation, two training phases, and testing (see supplementary material for detailed procedures). The judgment bias test consisted of a multimodal approach using location (spatial) and color (visual) as cues. Prior to any task, two chickens per pen were placed in a holding pen of 4.37 m^2. This had two functions, 1) it allowed the researcher to prepare the test arena for the judgment bias tasks after chickens were collected from the home pen, and 2) it allowed chickens to remain calm or calm down in a familiar environment comparable to the home pen, with a familiar conspecific, and with access to shavings, one feeder, and one water line. Food and water were provided *ad libitum*. One researcher performed all procedures.

Test arena

All procedures were performed in an arena made from plywood (91 cm L × 61 cm W) with yellow rubber interlocking mats (QC-Eb18N, Jiasheng, China) as flooring, two LED light bars, and a start box (30 cm L × 61 cm W) with access to the test area through a guillotine door (Supplementary Fig. Sl). A camera (Teledyne Flir LCC, OR, USA) was placed overhead to record all judgment bias procedures. All tasks were conducted between 8:30-13:00 h.

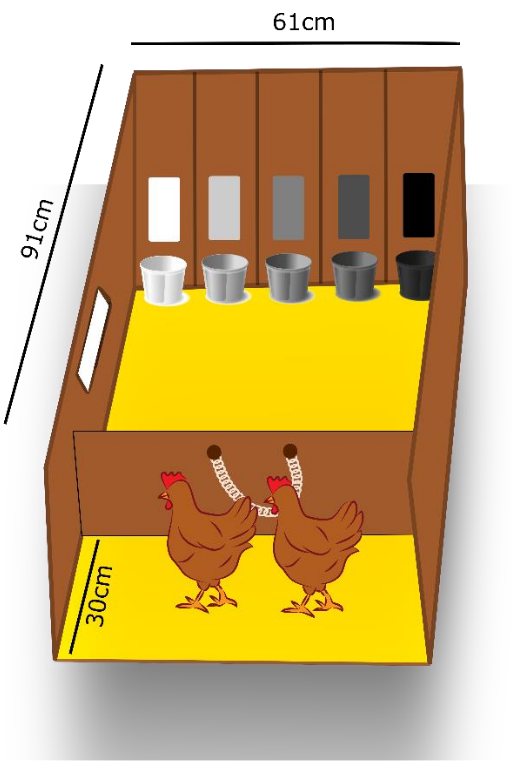

Figure S1. Judgment bias test arena. Chickens were placed in the start box prior to all training or testing sessions. Colored cues and associated containers (black or white) were placed at either reinforced location (left or right). During testing, three additional ambiguous-colored cues were placed at intermediate locations (near positive, middle, near neutral). During all training and testing phases, cues were individually presented.

Habituation

Chickens were first habituated to the judgment bias arena from 24 to 27 days of age (Supplementary Fig. S2). Chickens were habituated in pairs four times in the arena containing three arbitrarily-placed cardboard feed flats (5 × 5 cm) filled with dried mealworms and three empty transparent plastic bowls (120 ml; Ziploc®, S.C. Johnson & Son, Inc.). In the first round, chickens were placed directly into the test area with the start box door closed for 3 min while the observer was out of the chickens' line of sight. In the second round, chickens were placed into the start box with the guillotine door open for 5 min while the observer was out of the chickens' line of sight. In the third and fourth rounds, chickens were placed in the start box with the door closed. The door was then opened, and chickens could move freely for 5 min while the observer remained in the chickens' line of sight. Chickens were considered habituated when they consumed a mealworm during any habituation round. All chickens proceeded to the training phase.

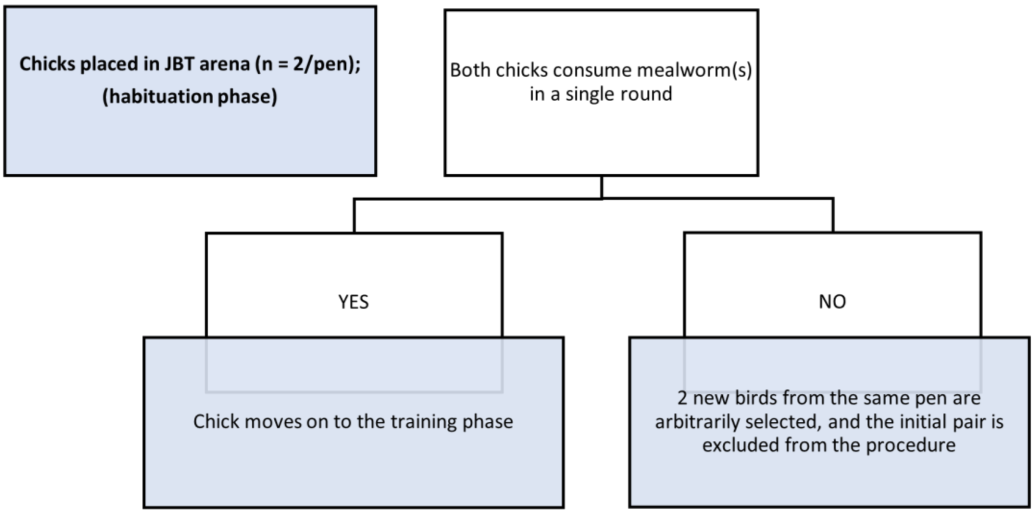

Figure S2. Diagram of the judgment bias habituation phase protocol (n = 24 chickens). All chicks undergo 4 habituation rounds. Blue boxes represent habituation phase methods, white boxes represent bird responses. Adapted from [59].

Training

The training was divided into phase 1 and 2 and was performed when chickens were between 30 to 51 days of age. Chickens were trained in pairs to associate a color cue of 100% black (n = 6) or white (n = 6) and location right (n = 6) or left (n = 6) with approximately 10 mealworms as a reward stimulus. Rewarded cues and locations were balanced across treatments. The color cue paper (25 cm L × 12 cm W) and a plastic bowl

(120 ml; Ziploc®, S.C. Johnson & Son, Inc.) of the same color were placed on or by the far wall of the arena, either at the far left or right depending on the color of the cue, with black rewarded cues always placed on the right and white rewarded cues always placed on the left. The opposite color and location represented the neutral stimulus, which consisted of a similar paper and bowl combination without a mealworm reward.

Training phase 1 was divided into two steps, A and B, and was response contingent. Depending on how chickens performed in the first step, they were assigned to step B or training phase 2 (Supplementary Fig. S3). During training phase 1A, paired chickens were presented with the positive cue and were required to peck it. Each training round lasted 3 min per pair, with 6 training attempts of 30 s per round. The chickens were given 30 s to respond to each attempt's positive cue. After each 30 s attempt, the observer gently picked up the chicken and placed it back into the start box to set up the arena for the next attempt. Unsuccessful attempts were followed by the observer immediately shaking and tilting the container. These rounds were repeated until chickens were considered learned (peck reward cue 5 out of 6 training attempts within a single round). If one or both chickens did not succeed in pecking at least 2 out of 6 times within the first round, they went to step B.

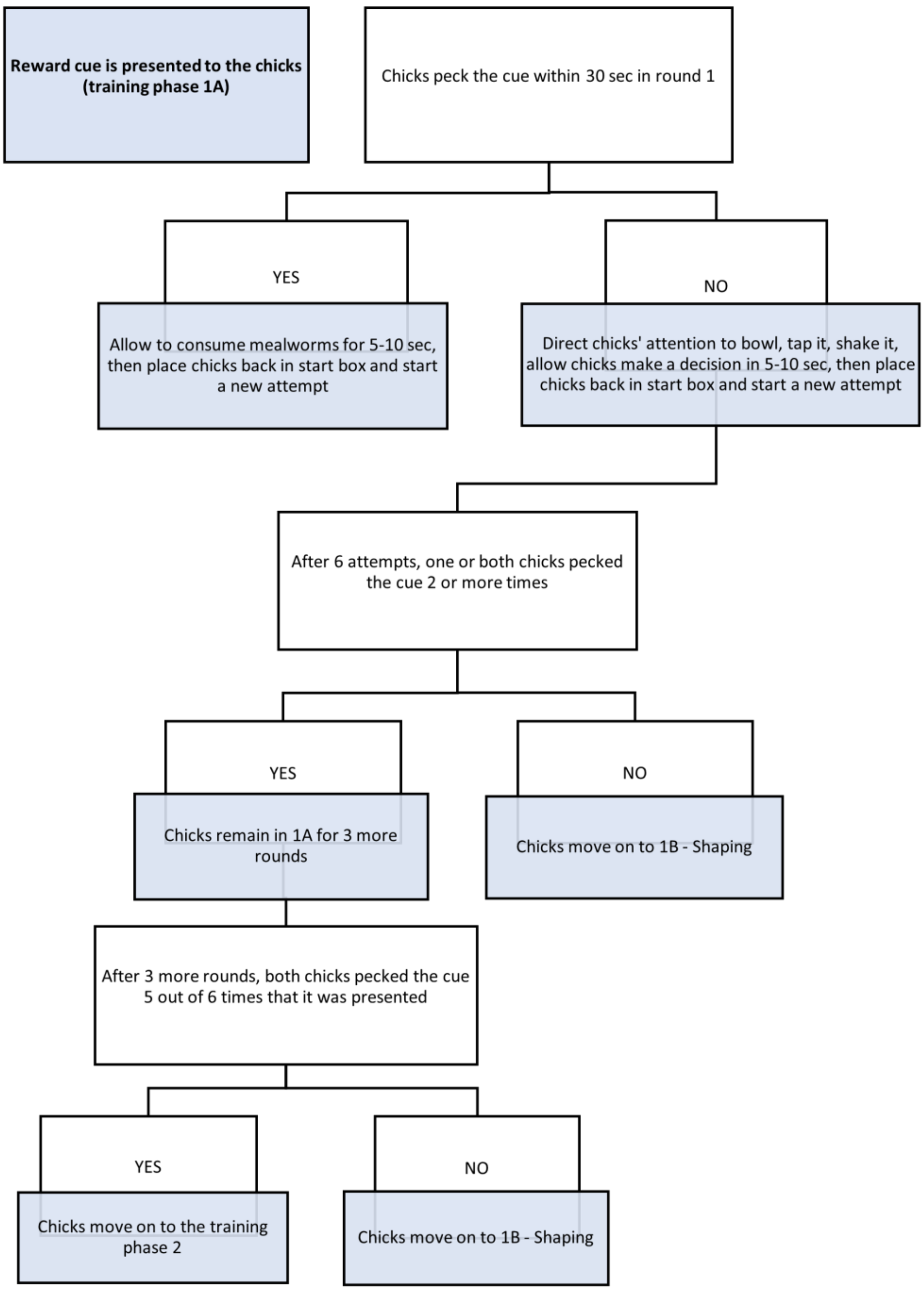

Figure S3. Diagram of the judgment bias training phase 1A protocol for round 1 (n = 24 chickens). Blue boxes represent training phase 1A methods, white boxes represent bird responses. Adapted from[59].

Training phase 1B (shaping) was used to allow those chickens a greater chance to reach the training criterion (Supplementary Fig. S4). During 3 rounds, a shaping procedure (Supplementary Fig. S5) was used until the rounds' learning criteria were met. After all rounds, the chickens that pecked cue 2 out of 6 training attempts within a single round were moved back to step A and remained until chickens were considered learned.

The chickens that pecked cue 5 out of 6 within a single round were moved to training phase 2. One pen (2 chickens) from the barren treatment was removed from the test because the animals did not meet with learning criterion.

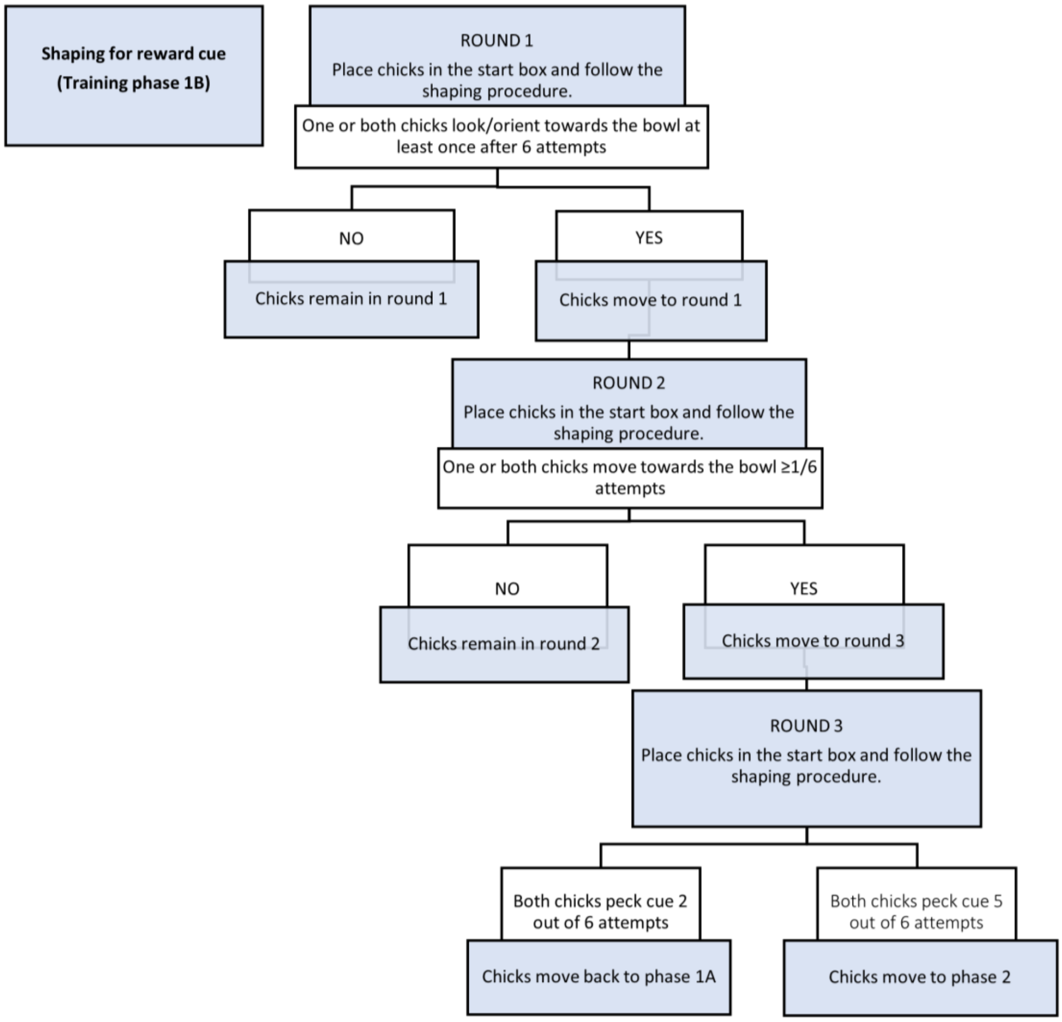

Figure S4. Diagram of the judgment bias training phase 1B protocol (shaping) (n = 24 chickens). Blue boxes represent training phase 1B methods, white boxes represent bird responses. Adapted from [59].

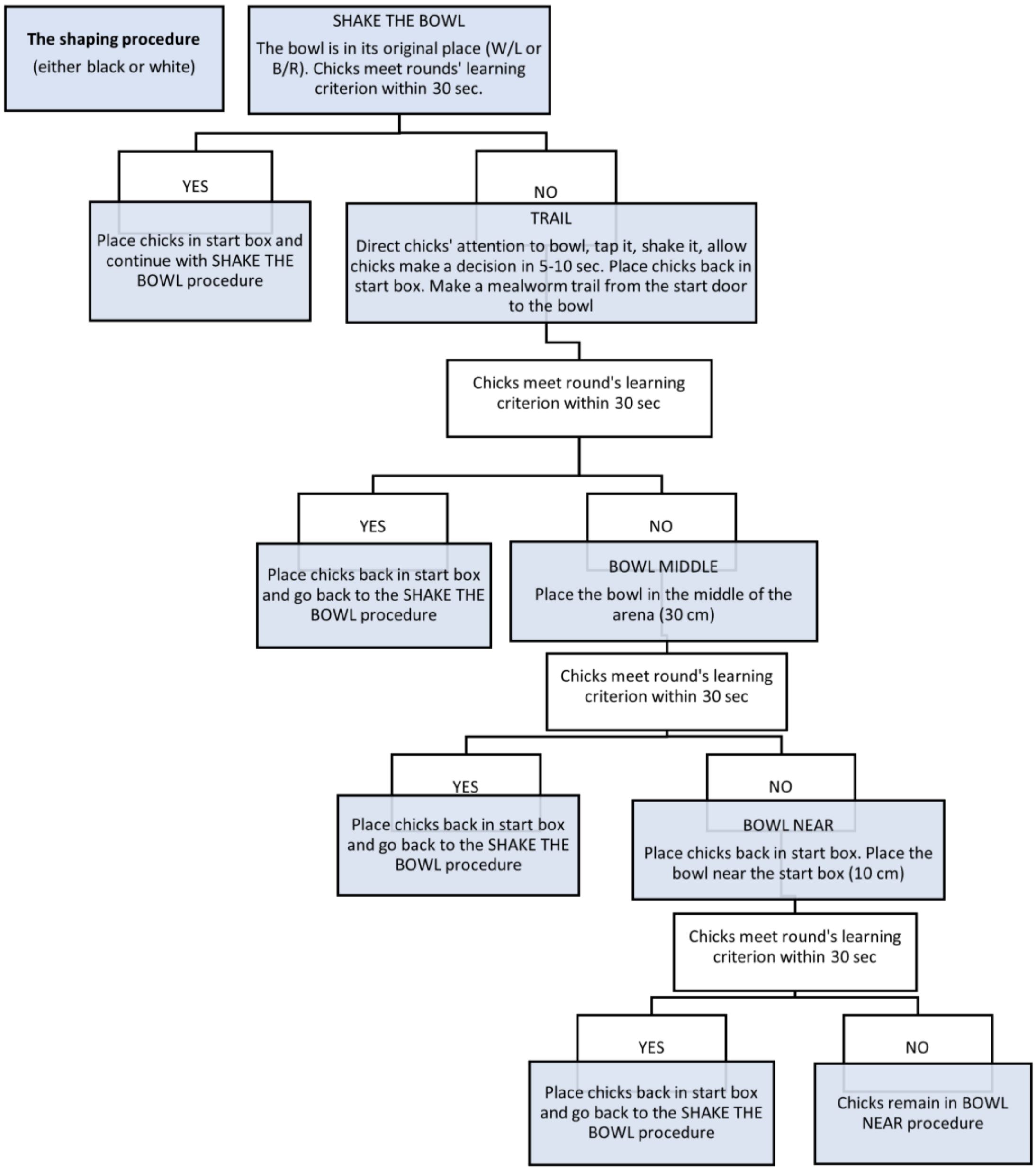

Figure S5. Diagram of the shaping procedure that was applied in each round of training phase 1B (n = 24 chickens). Learning criteria were orient (round 1), approach (round 2, or peck cue (round 3). Chicks will remain in this phase until successful, or the birds will be excluded from the trial after 7 rounds. Blue boxes represent shaping procedure methods, white boxes represent bird responses.

Training phase 2 (discrimination) began with 11 pens (n = 22, 2 chickens/ pen). Positive and neutral cues were presented individually according to a pseudorandomized order, with no more than two of either cue presented consecutively (Supplementary Fig. S6). Each training session lasted 180 s per group with six training attempts of 30 s. Phase

2 continued until at least one chicken met the training criterion (chickens must peck positive cue 100% of the time and not peck neutral cue 100% of the time they were presented into a single round). Pairs from 9 out of 11 pens (n = 18 chickens) met the training phase 2 learning criterion. In both pairs remaining, one chicken in each pair met the learning criterion. These two pairs moved on to the testing phase, but only the performance of chickens that met the learning criterion was recorded. All training took 13 days distributed across three weeks.

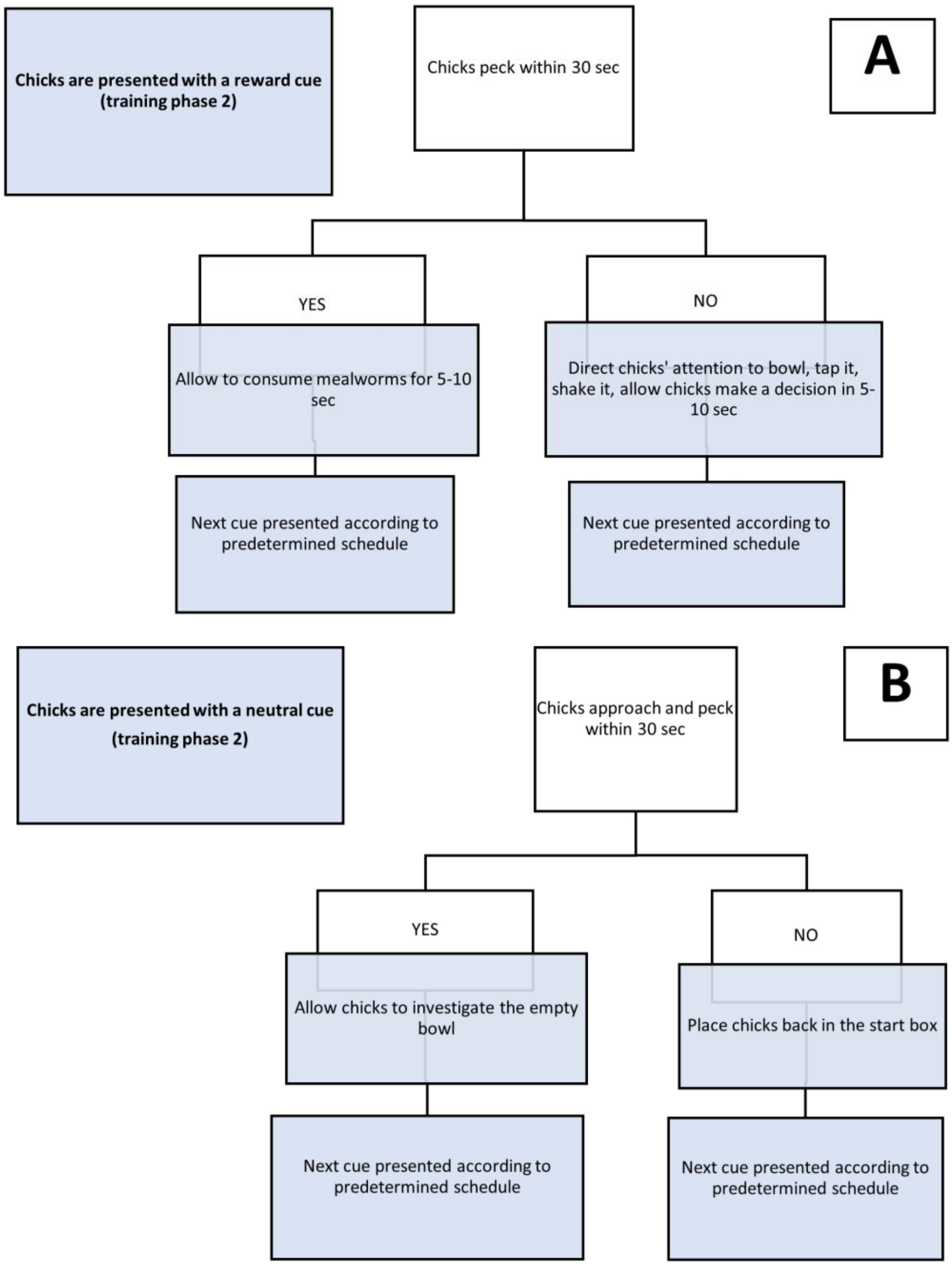

Figure S6. Diagram of the judgment bias training phase 2 protocol (n = 22 chickens). Blue boxes represent training phase 2 methods, white boxes represent bird responses. (A)

Chicks are presented with a reward cue. (B) Chicks are presented with a neutral cue. Adapted from [59].

Testing

The testing phase occurred in week 8 (days 52 and 53). All 11 pens (pairs) that advanced to testing (n = 20 chickens) were tested four times over two days. During testing, the Positive (P), Neutral (N), and three new, ambiguous cues, Near Positive (NP), Middle (M), and Near Neutral (NN), were individually presented with intermediate colors and at intermediate locations, 75% black (near right), 50% black (middle), and 25% black (near left) using a pseudorandomized order (Supplementary Fig. S7). Each testing session lasted a maximum of 7 min, with 1-min attempts for the chickens to approach each presented cue (in total, 28 attempts/pair). The first and last attempts in a testing session were always rewarded for maintaining motivation throughout the test. The researcher live-recorded frequency and latency to approach and proportion of chickens pecking cues. When one or both chickens did not approach or peck the cue, a maximum latency of 60 s was recorded.

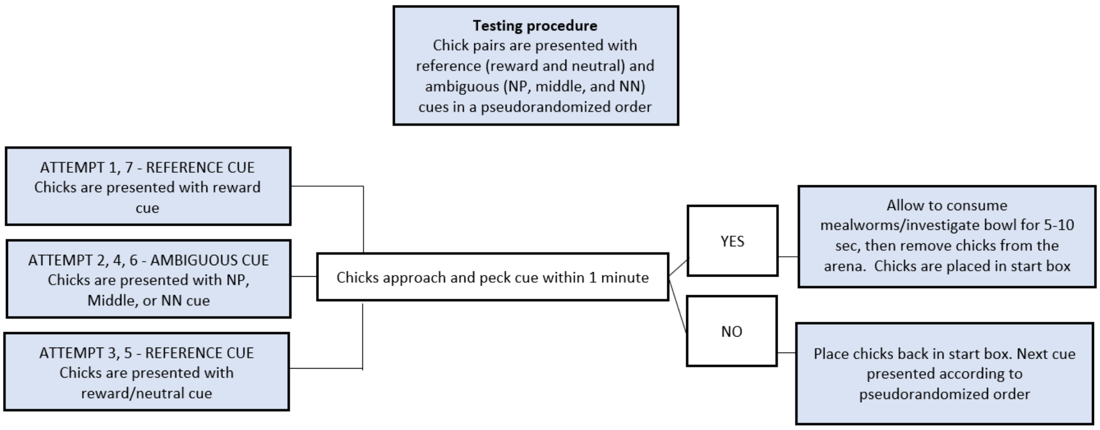

Figure S7. Diagram of the judgment bias testing phase protocol (n = 20 chickens). Blue boxes represent testing phase methods, white boxes represent bird responses. Each round has seven 1-minute attempts with reference cues (reward and neutral) and ambiguous cues (Near-positive [NP]; Middle; Near-neutral [NN]) being presented individually.

Lameness (gait)

The gait of all tested chickens was assessed by a trained observer when chickens were 53 days of age. A six-point scale was applied to classify gait scores[36]: score 0 normal walking, score 1 the chicken moves fast, but a slight walking deficiency is observed, score 2 the chicken moves fast, but there is a significant walking deficiency, score 3 the chicken moves fast, but it presents an important walking deficiency, score 4 the chicken moves with a serious difficulty, and score 5 the chicken barely moves and often uses its wings during locomotion.

Chronic stress

After the testing phase (day 53), three wing feathers were cut from all tested chickens to assess feather corticosterone concentrations as described in [37]. Feather weight was recorded. Feathers were minced into pieces (<5 mm), then HPLC-grade methanol (10 mL) was added. The samples were placed in a sonicating water bath at room temperature for 30 min, incubated in a shaking water bath at 50°C overnight, and methanol was separated from the feather material through a vacuum filter. After that, the original sample vial and filtration material were washed twice with 2.5 mL of methanol and added to the original methanol extract to avoid losing any corticosterone from the sample. The methanol extract was placed in under at the fume hood until the methanol had evaporated. The extracted residues were reconstituted in a small volume of the phosphate buffer system. A commercially available ELISA (Cayman Chemical Company, MI, USA) was performed according to the manufacturer protocol to quantify the concentration of feather corticosterone by mm and mg of feather material.

Behavioral observations

A tonic immobility test and attention bias test were performed in order to categorize chickens as fearful or anxious. The tonic immobility test assessed fearfulness[38,39] and the attention bias test assessed anxiety[39,40] when birds were 63 and 64 days of age, 10-11 days after the JBT testing phase was completed. Both tests lasted 300 s, and birds were categorized by relative fearfulness and anxiety using the median latency to righten and latency to begin feeding, respectively. When latencies were higher than the median, chickens were categorized as fearful or anxious. When latencies were below the median, chickens were categorized as fearless or calm (Supplementary Table S1).

Tonic immobility test

Chickens were subjected to a tonic immobility test, following the methodology described by[39]. Chickens were individually placed on their backs in a V-shaped wooden cradle with their heads hanging over the edge. The observer induced tonic immobility by placing two fingers on the bird's sternum and applying gentle pressure while covering the bird's head with the other hand for 15 s. Thereafter, the observer removed both hands. If a chicken rightened within 10 s after releasing, tonic immobility was reattempted. If tonic immobility was not induced after three attempts, the chicken was returned to their pen with their latency to righten recorded as 0 s.

Attention bias test

The attention bias test (ABT) was performed, following the methodology described by [39]. A square arena (125 cm W × 125 cm L× 91.4 cm H) was used with pine shavings on the floor, and a feeder containing commercial feed and mealworms. After each pair of chickens was placed in the arena, together with a third arbitrarily selected bird from the same pen as described by [39], an 8 s conspecific alarm call was played to elicit a vigilance response. Latency to begin feeding (s), latency to first vocalization (s), and occurrence (yes/no) of vigilance behaviors in the first 30 s following the first alarm call (visibly stretching neck, looking around, freezing, and erect posture)[5,40] were recorded. The alarm call wasreplayed and latency to resume feeding was recorded depending on birds' responses, as described by[39].

Table S1. Mean estimate responses and proportions (%) of broiler chickens characterized as fearful or fearless in the tonic immobility test (s ± SEM; 0-300 s) and broiler chickens characterized as anxious or calm in the attention bias test (s ± SEM; 0-300 s)

Tonic immobility test	Characterized relative personality[1]		
	Fearful	Fearless	n
Latency to righten (s)	165.4 ± 26.7	39.4 ± 5.9	22

Attention bias test	Anxious	Calm	n
Begin feeding (s)	174.4 ± 27.9	19.6 ± 5.9	22
First vocalization (s)	8.8 ± 2.8	18.9 ± 10.5	19
Resume feeding (s)	26.4 ± 8.9	25.4 ± 7.6	16
Vigilance behaviors			

Erect posture (% of chickens)	0.00	0.00	22
Neck stretching (% of chickens)	12.50	0.00	22
Looking around (% of chickens)	75.00	27.27	22
Freezing (% of chickens)	50.00	0.00	22

[1]Behavioral characterizations were done based on median scores of latency to righten in the tonic immobility test (fearfulness) and latency to begin feeding in the attention bias test (anxiety).

Statistical analysis

Data were analyzed in SAS Studio 3.8 (SAS Institute Inc., Cary, NC, USA). The variance homogeneity was assessed using Levene's test and data residual normality was verified by the Shapiro-Wilk's test. The distribution of data residuals and subsequent statistical approaches are shown in Table 1. Even though residuals were not normally distributed for latency to approach (Table 1), the use of mixed-effects models is appropriate as these are largely robust even to quite severe violations of model assumptions such as the residuals' distribution[41]. Model assumptions for skewness (0.136) and equal group variances (Levene's test $P = 0.648$) were met[42,43]. Generalized linear mixed models (GLIMMIX) were followed by F-tests or Tukey's multiple comparison tests. Correlations between chronic stress and fearfulness, chronic stress and anxiety, and anxiety and fearfulness were assessed using Pearson's correlation analysis with the CORR procedure. Associations were considered significant at $P < 0.05$ and a trend at $P < 0.1$.

Table 1. Statistical approaches per measurement, with response variable tested, distribution of data residuals, statistical test used, predictors that were assessed, and random variables that were included in the model.

Analysis	Measurement	Response variable (unit)	Distribution of data residuals	Test	Predictors tested in the model	Random variables included
Univariate	Judgment bias training	Training rounds (n)	Other	Wilcoxon rank-sum	Treatment	n/a[2]
					Fear	
					Anxiety	
					Chronic stress	
	Judgment bias test	Latency to approach (s)	Other	GLIMMIX[1]	Gait	Pen (bird ID), round, treatment, cue type, reward color/side
					Fear	
					Anxiety	
					Chronic stress	
					Reward color/location	Pen (bird ID), gait, round
		Chickens that pecked cues (%)	Binary		Gait	Pen (bird ID), round, treatment, cue type, reward color/side
					Fear	
					Anxiety	
					Chronic stress	
					Reward color/location	Pen (bird ID), gait, round
	Tonic immobility	Latency to righten (s)	Other	Wilcoxon rank-sum	Treatment	n/a
	Attention bias	Latency to begin feeding (s)				
		Latency to first vocalization (s)				
		Latency to resume feeding (s)				
		Vigilance behaviors (%)				
	Gait	Score (0-5)				
	Chronic stress	Feather corticosterone concentration (µg/mg)	Normal	GLIMMIX		Pen (bird ID)
Multivariate	Judgment bias test	Latency to approach (s)	Other	GLIMMIX	Treatment, cue type, and their interaction	Pen (bird ID), round, gait, cue color/side
		Chickens that pecked cues (%)	Binary			

[1] Generalized linear mixed models [2] Not applicable.

Results

Judgment bias test

Habituation

One pair of chickens from the low-complexity treatment did not eat mealworms throughout the 4 habituation rounds. Therefore, they were omitted from the experimental procedures and replaced with a new arbitrarily selected pair from the same pen. During habituation round 1, 25% of chickens consumed at least 1 mealworm (latency mean ± SD: 127 ± 17 s), 75% in round 2 (97 ± 24 s), 96% in round 3 (36 ± 11 s), and 100% in round 4 (18 ± 7 s).

Training

In training phase 1A (conditioning for reward cue), chickens from 6 (2 low-, 4 high-complexity pens) out of 12 pens met the learning criterion and proceeded to training phase 2 (Supplementary Table S2). The unsuccessful chickens from the other 6 pens (4 low-, 2 high-complexity pens) were moved to training phase 1B (shaping for reward cue).

Chickens from 5 pens (2 low-, 3 high-complexity pens) took between 2 and 7 rounds to meet the 1B learning criterion. Two chickens from a single low-complexity pen were omitted from the experimental procedures because they did not meet the phase 1B learning criterion after 7 rounds.

In training phase 2 (discrimination between reward and neutral cue), chickens from 9 out of 11 pens took between 1 and 7 rounds (median of 2 rounds/pen) to meet the learning criterion. One of two chickens from each remaining pen (1 low-, 1 high-complexity pen) met the criterion, while the other did not meet the learning criterion after 7 rounds. Complexity treatment, fearfulness, anxiety, and chronic stress did not impact learning success (number of rounds needed to meet training criteria) for any training phase or overall (Supplementary Table S2). By the end of training phase 2, 83% of chickens (20 out of 24) successfully met the learning criterion.

Table S2. Number of successful chickens (n) and JBT training rounds (mean ± SD) needed to meet the learning criteria by complexity treatment, behavioral and chronic stress categorization.

Training phase	1A[1]		1B[2]		2[3]		Total		Statistical test and *P*-value[4]
	Round n	Bird n	Round n	Bird n	Round n	Bird n	Round n	Bird n	
Complexity treatment									
Low-complexity	2 ± 1	4	2 ± 1	8	2 ± 1	9	5 ± 2	9	χ^2 = 1.00, *P* = 0.317
High-complexity	2 ± 1	8	3 ± 1	4	2 ± 2	11	4 ± 2	11	
Behavioral characterization									
Fearful	2 ± 1	7	3 ± 1	5	1 ± 1	10	4 ± 2	10	χ^2 = 1.69, *P* = 0.193
Fearless	1 ± 1	5	2 ± 1	7	2 ± 2	10	5 ± 3	10	
Anxious	2 ± 1	7	3 ± 1	5	1 ± 1	10	4 ± 3	10	χ^2 = 0.53, *P* = 0.467
Calm	1 ± 1	5	2 ± 1	7	2 ± 2	10	5 ± 2	10	
Chronic stress									
Low-stressed	1 ± 1	7	3 ± 0	2	2 ± 2	9	4 ± 3	9	χ^2 = 0.72, *P* = 0.397
High-stressed	2 ± 1	5	2 ± 1	6	2 ± 1	11	5 ± 2	11	

[1]Conditioning for reward cue

[2]Shaping for reward cue

[3]Discrimination between reward and neutral cue

[4]Univariate analysis for predictors (treatments, personality traits, chronic stress categorization) on total learning success (n rounds needed to reach learning criteria)

Testing

Fear, anxiety, chronic stress, and gait score did not impact JBT responses (latency to approach or proportion of chickens pecking cues; $P \geq 0.180$). Test round did not impact the latency to approach ($F_{3,287}$ = 0.54; P = 0.654) or proportion of chickens that pecked cues ($F_{3,287}$ = 0.38; P =0.766). Reward side (right and left) or color (black or white) did not impact the latency to approach ($F_{1,188}$ = 1.08; P = 0.301) or proportion of chickens that pecked cues ($F_{1,185}$ = 0.33; P = 0.564).

Regardless of treatments, chickens successfully discriminated between cues, as they approached the reward cue faster than near-positive (NP; P = 0.008), middle (P < 0.001), near-negative (NN; P < 0.001), and neutral (P <0.0010 cues, they approached the NP cue faster than NN (P < 0.001) and neutral cues (P < 0.001), and approached the middle cue faster than the NN (P = 0.002) and neutral (P < 0.001) cues ($F_{4,287}$ = 78.16; P

< 0.001; Fig. 2). Similarly, more chickens pecked the reward cue compared to NP (P < 0.001), middle (P < 0.001), NN (P < 0.001), and neutral (P < 0.001) cues, and more chickens pecked the middle cue compared to NN (P = 0.016) and neutral (P = 0.007) cues ($F_{4,287}$ = 46.33; P < 0.001; Fig. 3).

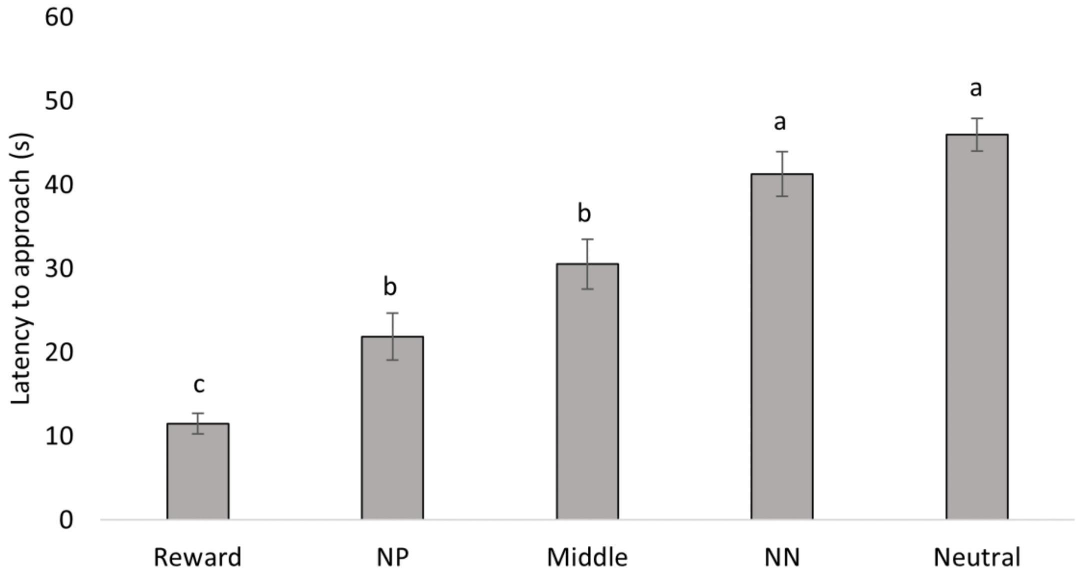

Figure 2. Mean (± SEM) latency to approach (s) all five cues (Reward; Near-positive [NP]; Middle; Near-neutral [NN]; and Neutral) in 4 rounds of the judgment bias test (n = 11 social pairs). Means with uncommon superscripts ($^{a–c}$) differ at P < 0.001.

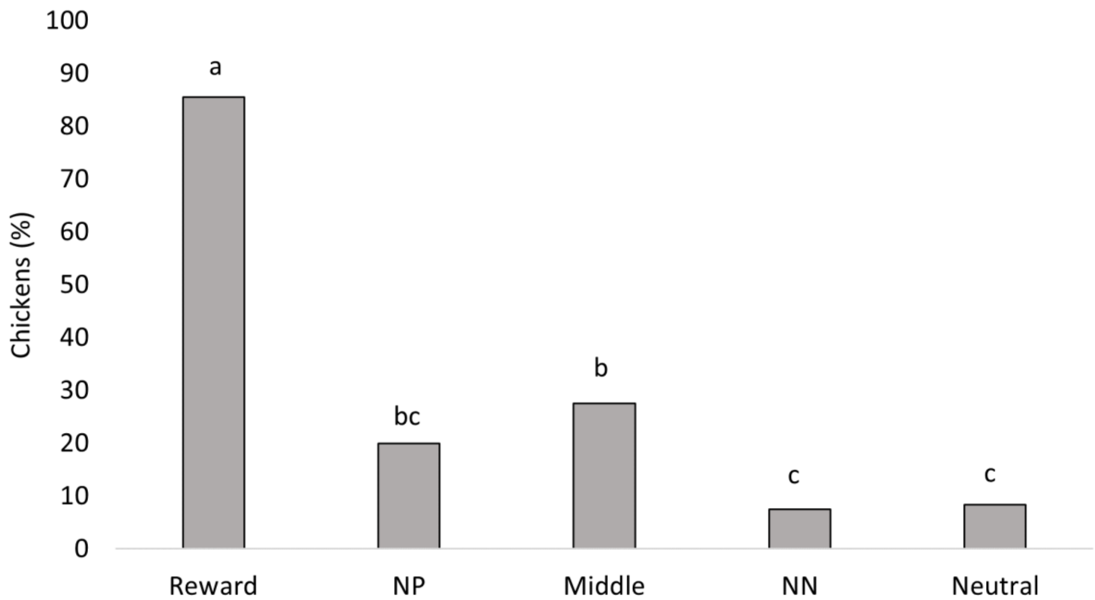

Figure 3. Proportion (%) of chickens that pecked all five cues (Reward; Near-positive [NP]; Middle; Near-neutral [NN]; and Neutral) in 4 rounds of the judgment bias test (n = 11 social pairs). Proportions with uncommon superscripts ($^{a–c}$) differ at P < 0.001.

An interaction effect between treatment and cue type was found for latencies to approach cues ($F_{4,287}$ = 3.56; P = 0.029, Fig. 4). Chickens from the low-complexity treatment approached the middle cue faster than chickens from the high-complexity treatment (P = 0.014), but no differences between complexity treatments were found for the reward (P = 0.992), NP (P = 0.951), NN (P = 0.660), and neutral (P = 0.926) cues. Chickens from the low-complexity treatment tended to approach cues faster than those from the high-complexity treatment ($F_{1,287}$ = 3.13; P = 0.077). More chickens in the low-complexity treatment (45%) tended to peck cues than chickens in the high-complexity treatment (35%, $F_{4,287}$ = 3.22; P < 0.073). No interaction between complexity treatment and cue type was found for the proportion of chickens pecking the cues ($F_{4,287}$ = 2.15; P = 0.174).

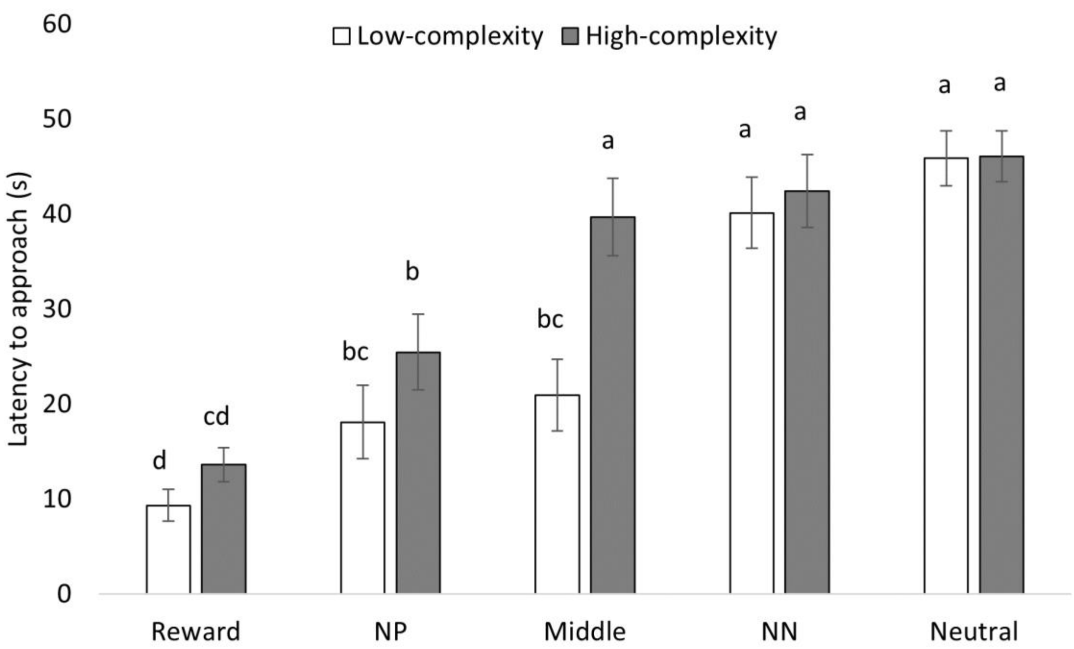

Figure 4. Mean (± SEM) of latency to approach (s) all five cues (Reward; Near-positive [NP]; Middle; Near-neutral [NN]; and Neutral) in 4 rounds of the judgment bias test by complexity treatment (n = 11 social pairs). Means with uncommon superscripts ($^{a–d}$) differ at P < 0.05.

Lameness (gait)

The environmental complexity treatment did not affect gait score (χ^2 = 0.20, P = 0.888), with 66.7% of chickens from the low-complexity treatment receiving a score 0 and 33.3% a score 1, and 63.6% of chickens from the high-complexity receiving a score 0 and 36.4% receiving a score 1.

Behavioral observations

The environmental complexity treatment did not impact latency to righten in the tonic immobility test (Supplementary Table 3). In the attention bias test, more chickens in the low-complexity treatment performed vigilance behaviors such as looking around and freezing compared to chickens in the high-complexity treatment (Supplementary Table 3). Complexity treatment did not impact the proportion of chickens performing neck stretching, or latencies to first vocalization, to begin feeding, or to resume feeding (Supplementary Table 3). Erect postures were not observed during the attention bias test.

Table S3. Least squares mean estimates (± SEM) and proportions (%) of broiler chickens from low- or high-complexity treatments in the tonic immobility test (0-300 s) and the attention bias test (0-300 s)

Personality trait test responses	Complexity treatments			Test statistic and P-value for complexity effect
	Low-complexity	High-complexity	Bird n	
Tonic immobility test				
Latency to righten (s)	113 ± 33	84 ± 19	22	$\chi^2 = 0.31$, $P = 0.575$
Attention bias test				
First vocalization (s)	11 ± 3	15.2 ± 9	19	$\chi^2 = 0.51$, $P = 0.476$
Begin feeding (s)	128 ± 35	70 ± 26	22	$\chi^2 = 2.31$, $P = 0.128$
Resume feeding (s)	25 ± 9	26 ± 7	16	$\chi^2 = 0.01$, $P = 0.957$
Erect posture (% of chickens)	0	0	22	n/a[1]
Neck stretching (% of chickens)	11	0	22	$\chi^2 = 1.11$, $P = 0.292$
Looking around (% of chickens)	77	20	22	$\chi^2 = 6.01$, $P = 0.014$
Freezing (% of chickens)	44	0	22	$\chi^2 = 5.33$, $P = 0.021$

[1]No statistical analysis was performed because behavior was not observed.

Chronic stress

Feather corticosterone concentrations tended to be higher in chickens from the high-complexity treatment than those from the low-complexity treatment ($F_{1,19} = 3.63$; $P = 0.071$, Fig. 5). No correlations were found between chronic stress and fearfulness (r =

0.310, P = 0.161), chronic stress and anxiety (r = 0.154, P = 0.494), or anxiety and fearfulness (r = 0.333, P = 0.123).

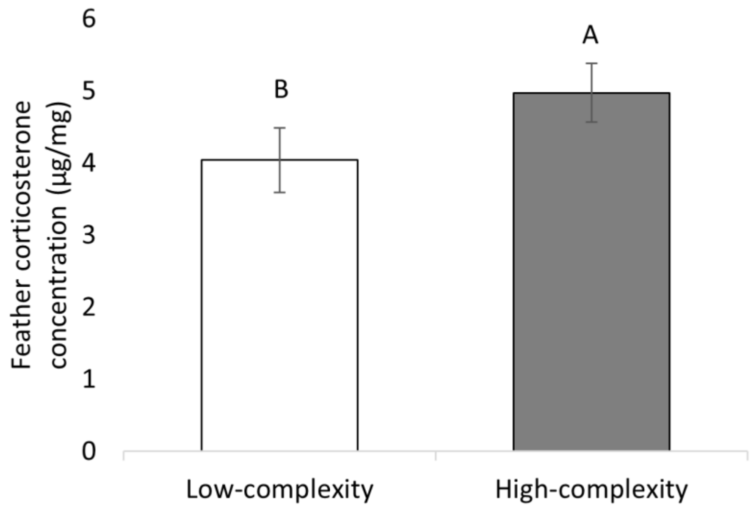

Figure 5. Least square mean estimates (± SEM) of feather corticosterone concentrations for chickens from low- or high-complexity treatments (n = 22 chickens). Means with uncommon superscripts ($^{A\text{-}B}$) differ at P < 0.1.

Discussion

This is the first study to apply a social-pair training and testing approach in a judgment bias task. We inferred the affective state of slow-growing broiler chickens housed in low- or high-complexity environments from behavioral responses in the JBT. In addition, we evaluated the effects of fearfulness, anxiety, and chronic stress (feather corticosterone concentration) on JBT training and testing performance. During 13 days of training across three weeks, 83% of chickens were successfully trained to discriminate between multimodal reinforced cues. Chickens showed a generalization gradient response in the JBT, demonstrating that chickens successfully learned the discrimination task[3,5,12]. All chickens approached and pecked ambiguous cues close to the reward cue more quickly or more often than cues close to the neutral cue, without showing a color or side bias. This suggests a greater expectation of a reward for ambiguous cues near the reward cue. Based on these findings, a social-pair JBT approach can be used as a tool to infer affective states in slow-growing broiler chickens.

Complexity treatment, fearfulness, anxiety, and chronic stress did not impact the number of rounds chickens needed to meet the learning criterium for each training phase.

Training chickens in pairs may have attenuated the effects of social isolation and novelty inherent to the JBT environment, especially for chickens with negatively valenced affective states. Studies using individual training approaches showed that stress and fearfulness negatively impacted laying hens' and fast-growing broilers' training performance in cognitive tests[19,26,44]. Broilers that were stressed by social isolation showed impaired spatial memory learning compared to a control group[19]. The authors argued that chronically stressed broilers were less capable of coping with negative conditions associated with the test (isolation and novelty)[45,46], sensitizing them to respond to future stressful events (more testing) and provoking a shift in cognitive functions away from spatial learning and toward a stress response[19,47]. Similarly, rats and mice chronically stressed by social isolation showed impaired spatial learning in a water maze test compared to a control group[48,49]. Fearful rats made more side errors in Y-maze[50] and water maze tasks[51], while anxious rats showed poor learning performance in a water maze task[52–55] compared to their control group. Furthermore, fearful, stress-sensitive laying hens developed side biases during JBT training[26,44]. The authors argued that fearful hens use a rigid response strategy during early learning phases by choosing a specific side repeatedly irrespective of success, indicating cognitive inflexibility[26,44]. In the current study, no such effects of fearfulness, anxiety, or chronic stress were observed on learning success or test responses.

The social-pair testing approach may have attenuated the negative effects of negatively valenced fearfulness, anxiety, and chronic stress. During testing, broilers experienced social support from a flock mate, which could increase their motivation to perform tasks. Broilers have a strong motivation for social reinstatement and chickens in natural settings live in relatively small, highly social groups[56–58]. In line with that, laying hens exhibited less fear-related behaviors when undergoing an open field test[59] and fast-growing broilers performed better in an attention bias test when tested with two conspecifics compared to being tested alone[39]. These results suggest that chickens benefit from social support in testing environments that require learning or attention[15]. Our social-pair testing approach could have been especially beneficial for fearful, anxious, or chronically stressed animals, reflected in their similar learning performance compared to birds that were considered fearless, calm, or experiencing less chronic stress.

The learning success rate (20/24 chickens) in this study was greater than reported in earlier studies using an individual approach for fast-growing broilers and laying hens[5,7,9,12,13,60], but lower than reported in[14]. Days needed to train birds were comparable to or faster than most other JBT studies. Training took between 10-30 days for fast-growing broilers, with low learning success rates (between 25 and 51%)[5,9], and training took between 13 days and 8 weeks for laying hens, with better learning success rates (between 62 and 100%)[6,7,12,13]. However, all genetic strains differed from the strain used in the current study, which could influence the result.

We theorize that social facilitation improved chickens' learning ability. Social learning helps chickens to decide what to eat and avoid[15]. Aversive behavior of one chicken towards a food item will result in consistent avoidance of that food item in an observing chicken[61]. As training phases 1A and 1B required birds to peck a reward cue with a food item, social learning (one bird observing another) could have contributed to chickens learning to peck the reward cue containing attractive food items. Furthermore, the paired approach could have facilitated spatial memory development and cue discrimination ability in training phase 2, as young chickens can locate hidden objects due to their developed spatial memory[62], which allows them to learn from conspecifics through observation[63].

The benefit of a social training approach could differ depending on genetic strain, yet in the current study only a slow-growing broiler strain was tested. Inherent stressors associated with JBT training and testing are the frequent handling by and close proximity with humans, plus repeated removal from home environments and flock mates, which could result in chronic stress[64]. These JBT procedures may be more distressing to slow-growing broilers than fast-growing broilers, as slow-growing broilers are more reactive to human interaction[65–69]. The improved training success compared to previous studies could suggest that the presence of a conspecific alleviated some of these negative experiences, thus a social approach may be especially beneficial for slow-growing broiler chickens. As chickens are a social species, this benefit is expected for other genetic strains too. Further research on social approaches in other genetic strains can confirm this.

Chickens from the low-complexity treatment were faster to approach all cues and the middle cue compared to chickens from the high-complexity treatment. Furthermore, more chickens from the low-complexity treatment tended to peck cues than those from the high-complexity treatment. These differences indicate that chickens from simple environments were more optimistic than chickens from enriched environments, in contrast with our hypothesis. One explanation could be related to the environmental enrichment used has been inappropriate for slow-growing broiler chickens and negatively impacted their affective state. Our enrichment strategy was to provide a complex and varied environment, maintaining environmental novelty and providing resources to fulfill highly-motivated behavioral needs. This highly complex environment effectively improved emotions and affective states in fast-growing broiler chickens[5,39]. However, slow-growing broiler chicken strains are more active and interact more with conspecifics and the environment than fast-growing strains[70,71], which could have negated the potential benefit of the chosen enrichment items. If the enrichments were unsuited for slow-growing broilers, they might have elicited frustration or other negative emotions, resulting in negative affective states. In line, a highly-complex environment increased chronic stress parameters in mice and corvids compared to animals kept in barren environments[72–74]. Further supporting this theory and previous research findings, the chickens from the high-complexity treatment tended to show an increased chronic stress response compared to chickens in the low-complexity treatment. The increased chronic stress response in high-complexity chickens could in part be due to the increased human-animal interactions associated with providing temporary enrichments, since slow-growing broilers are more reactive to human interaction than fast-growing strains[65–69]. Alternatively, the novelty of these enrichments, assuming that novelty was maintained, might have increased birds' arousal and thus increased corticosterone deposition in feathers. Increased arousal can increase circulating corticosterone concentrations, also when animals experience a positively valenced emotion such as pleasure, excitement, and winning[75–77]. As the majority of research on environmental enrichments for broiler chickens is focused on fast-growing strains[78], we recommend further research assessing slow-growing broilers' preferences for environmental enrichments.

In the current study, complexity treatments did not affect gait score, with gait being perfect or slightly deficient for all birds assessed. In line, slow-growing broilers generally have good walking ability[66,79]. Latency to approach cues and proportion of chickens pecking cues were not impacted by gait, in line with fast-growing broilers in a JBT[5]. These results suggest that observed differences in latencies and proportion of chickens approaching were reflecting a cognitive bias instead of physical limitations to approach the cues.

In order to avoid the effects of fear and anxiety tests on JBT responses, we performed these tests ten days after the JBT testing phase. As life experiences shape behavioral responses and individual preferences over time[27], the JBT procedure could have impacted the chickens' behavioral responses on fear and anxiety tests. Repeated training and testing could have reduced fear towards humans as chickens habituated to repeated handling[80]. This study design did not allow us to assess a balanced sample of fearful, fearless, anxious, and calm chickens in both complexity treatments. In addition, our limited sample size may have reduced the statistical power to assess the effects of fearfulness, anxiety, and chronic stress on training performance. We recommend further research assessing fear, anxiety, and chronic stress with a larger sample size to confirm the lack of impact found in the current study. Furthermore, this study did not incorporate a control group to directly compare a social-pair JBT approach with an individual JBT approach due to time constraints. Yet, even without a direct comparison, the social approach seems to result in improved learning ability compared to training success when birds are tested individually[5,7,9,12,13,60].

To conclude, this study is the first to show that a social-pair judgment bias training and testing approach can be used to successfully assess affective states in slow-growing broiler chickens, with no effects of fearfulness, anxiety, or relative chronic stress (based on feather corticosterone concentrations) on the chickens' learning ability during training or testing. The judgment bias test in this study showed that slow-growing broilers housed in unenriched, low-complexity environments were more optimistic to receive a reward in an ambiguous situation than broilers from an enriched, high-complexity environment. We recommend further studies directly comparing individual and social-pair JBT approaches. Chickens from the high-complexity treatment tended to show an increased chronic stress response and a more negative affective state compared to the chickens in the low-complexity treatment, possibly related to the suitability of provided environmental enrichments.

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CHAPTER 4

IMPLICATIONS

Conducting this study brought important information regarding the impacts of using environmental enrichment on fast- and slow-growing broiler chickens' welfare. With lameness still being the main welfare problem in poultry and with the evolution of animal welfare science recognizing that animal welfare is related to the individual and its experiences throughout life, the papers generated from this doctoral thesis will significantly contribute to the literature of the area.

This study was the first in literature to assess environmental enrichment effects on subclinical spondylolisthesis prevalence. Step platforms and laser lights were effective in improving chickens’ locomotor systems and reducing subclinical spondylolisthesis prevalence compared to the control group. Despite not reducing subclinical spondylolisthesis prevalence, hay bales increased the frequency of exploratory behaviors, breaking the environment monotony and providing opportunities for chickens to express species-specific behaviors. The use of the three environmental enrichment resources maintained chickens’ performance, showing to be viable for commercial application.

This thesis also resulted in the development of a new judgment bias test for slow-growing broilers, aiming to measure affective states from a social-pair approach, unprecedented in literature. Based on the social aspects of chickens that impact cognitive processes such as learning, the new judgment bias test uses conspecific presence during all training and testing phases. The results of this study showed that a new social-pair approach reduced fearfulness, anxiety, and chronic stress effects on training and testing performance, which have been reported in the literature as factors that prolong training phases.

Additionally, the findings showed that the environmental enrichment strategy used in this study, using permanent and temporary enrichments, was not appropriate for slow-growing broilers. This resulted in chickens tending to be more chronically stressed and with negative affective states compared to animals raised in a low-complexity environment. Several factors may contribute to this, such as the increase in human presence due to the rotation of temporary enrichments and the increase in the arousal levels of the animals due to environmental novelty maintenance.

From this thesis, more research can be developed to increase the understanding of environmental enrichment effects on subclinical spondylolisthesis prevalence, frequencies of the species' natural behaviors, and chickens’ affective states. In addition, there is a need to study and develop better environmental enrichment strategies for slow-growing broilers and to improve the judgment bias test for chickens, considering the social aspects of the species.