Chicken or fish? Do environmental complexity and stocking density impact affective states of broiler chickens and rainbow trout?

Mallory Grace Anderson

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Leonie Jacobs, Chair

Erica Feuerbacher

Gareth Arnott

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ACADEMIC ABSTRACT

In commercial settings, broiler chickens and rainbow trout are housed in barren environments under high stocking densities, due to an emphasis on production efficiency. These monotonous housing conditions do not provide broilers or trout with the ability to perform functional, highly-motivated behaviors and increase their susceptibility to excessive anxiety and fear, resulting in negative affective states and poor animal welfare. Affective state (or emotional state) is a cumulative product of short-term life experiences, ranging from positive to negative. Because affective states are largely influenced by environmental condition, determining animal affective state can provide useful information on how to improve housing conditions in order to ensure positive experiences and good animal welfare. Cognitive processes are closely associated with affective state; a "cognitive bias" occurs when affective state influences aspects of cognition, such as judgement and attention. Animals in positive affective states make optimistically-biased decisions during ambiguous situations, judging the situation as if it will produce a positive outcome, and show less bias towards a perceived threat, responding in a less anxious and calm manner. Animals in negative affective states make pessimistically-biased decisions during ambiguous situations, judging the situation as if it will result in a negative outcome. Additionally, animals in negative affective states will bias their attention towards a perceived threat rather than alternative stimuli, responding in an anxious manner. Therefore, judgement and attention bias tests can be used to determine animal affective states.

In Chapter 3, a judgement bias test was used to determine affective state of broiler chickens housed in either complex (perches, dust bath, pecking stones, and rotating enrichment objects) or barren (no enrichment) environments under either high or low

stocking densities. Broilers housed in complex environments responded more optimistically during the judgement bias test than broilers from barren environments, indicating the former were in a positive affective state. Stocking density did not impact their responses in the judgement bias test, indicating that affective states were not impacted by that treatment. In Chapter 4, an attention bias test was used to determine level of anxiety and a tonic immobility test was used to determine fear in order to investigate affective state of broilers housed in the same conditions as described for Chapter 3. Broilers housed in complex environments were less anxious during the attention bias test than broilers from barren environments, indicating environmental complexity reduced anxiety in broilers. Stocking density did not impact anxiety. Broilers from high stocking density environments had shorter tonic immobility durations than broilers from low stocking density environments, suggesting the former were less fearful. Environmental complexity did not impact fearfulness. In Chapter 5, a judgement bias test was used to determine affective state of rainbow trout housed in either complex (shelter structure and artificial plants) or barren (no enrichment) tanks under either low or high stocking densities. Trout housed in high stocking density tanks responded optimistically during the judgement bias test, indicating they were in a more positive affective state compared to trout housed in low stocking density tanks. Environmental complexity did not impact their responses in the judgement bias test, indicating no effect of enrichments on affective states was found.

These results indicate a beneficial relationship of a complex environment on broiler chicken affective state, observed through an optimistic judgement bias and reduced attention bias (anxiety) towards a perceived threat. Thus, providing a complex housing environment for broilers can improve their welfare and result in a positive affective state. Rainbow trout reared at the tested high density resulted in a positive affective state, although complexity did not benefit their welfare. Our results contribute much needed information on stocking densities to ensure fish welfare. Overall, environmental complexity, not stocking density, had a positive impact on broiler chicken affective states. Rainbow trout affective states were positively impacted by stocking density, but not environmental complexity.

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GENERAL AUDIENCE ABSTRACT

Conventional housing of broiler chickens and rainbow trout (both raised for meat) causes concern for their welfare and affective states. Environmental conditions can greatly impact animals' affective states–their long-term emotional state, ranging from positive to negative. In barren environments at high stocking densities, broiler chickens and rainbow trout are prevented from showing normal behaviors and these conditions can compromise their affective state and welfare. By 'asking' chickens and trout whether the glass is half full or half empty, we can determine level of optimism or pessimism, and level of anxiety or calmness, therefore gaining a better understanding of their affective states. This can be done using a judgement bias test and attention bias test, where animal responses (optimism and anxiety) are recorded during ambiguous situations (judgement) and threatening situations (attention). Animals in positive affective states judge ambiguous situations optimistically (glass half full) and pay little attention towards perceived threats, while animals in negative affective states judge the same ambiguous situations pessimistically (glass half empty) and pay more attention towards perceived threats.

In Chapter 3, responses to ambiguous situations were used to determine the affective state of broiler chickens housed in either enriched (perches, dust bath, pecking stones, rotating toys) or barren environments at either high or low stocking densities. Broiler chickens housed in enriched environments had an optimistic judgement bias of ambiguous situations (glass half full), suggesting they were in a more positive affective state compared to broilers housed in barren environments. Stocking density did not impact their level of optimism. In Chapter 4, responses to a perceived threat were used

to determine level of anxiety and a tonic immobility test was used to determine fear of broilers housed under the same conditions as in Chapter 3. Broilers housed in enriched environments paid less attention to a perceived threat than broilers housed in barren environments, indicating the former were less anxious (glass half full) and in a positive affective state. Fear was not impacted by the tested enrichments, but birds kept under higher stocking densities did show reduced fear compared to birds in low-density environments. In Chapter 5, rainbow trout were housed in either enriched (shelter structure and artificial plants) or barren tanks at either high or low stocking densities. Affective state was evaluated through their responses to ambiguous situations. Trout housed in high stocking density environments had an optimistic judgement bias of ambiguous situations (glass half full), suggesting they were in a more positive affective state than trout housed in low stocking density environments. The enrichments did not impact their responses during the test, suggesting they did not impact fish optimism.

These results indicate that an enriched environment improves broiler affective state and welfare compared to conventional housing conditions, the tested densities did not impact their welfare. Although an enriched environment did not positively impact responses of trout during ambiguous situations, our results show that housing rainbow trout in large groups results in a positive affective state and improved welfare status compared to housing trout in small groups. Overall, environmental enrichment, not stocking density, had a positive impact on broiler chicken affective states. Rainbow trout affective states were positively impacted by stocking density, but not environmental enrichment.

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TABLE OF CONTENTS

Chapter 1. Introduction **1**

Chapter 2. Literature review **5**
2.1 Broiler chickens 5
2.2 Rainbow trout 10
2.3 Environmental complexity 16
2.4 Affective state: Defining relevant terms 26
2.5 Quantifying affective state: Cognitive bias tests 29

Chapter 3. Environmental complexity, but not stocking density, positively impacts affective states of broiler chickens **42**
3.1 Abstract 42
3.2 Introduction 43
3.3 Results 47
3.4 Discussion 50
3.5 Materials and Methods 53

Chapter 4. Effect of environmental complexity and stocking density on fear and anxiety in broiler chickens **60**
4.1 Abstract 60
4.2 Introduction 61
4.3 Results 64
4.4 Discussion 70
4.5 Materials and Methods 75

Chapter 5. Impact of environmental complexity and stocking density on affective states of rainbow trout (Oncorhynchus mykiss) **86**
5.1 Abstract 86
5.2 Introduction 87
5.3 Results 89
5.4 Discussion 93
5.5 Materials and Methods 98

Chapter 6. Discussion and conclusions **108**
6.1 Recommendations for future research 110

Chapter 7. References **114**

Chapter 1. Introduction

Although there is not one universal definition of animal welfare, there is a general consensus that the definition should encompass an individual animal's experiences and how it perceives its environment[1,2]. For example, Broom (1986)[3] defines animal welfare as "its state with regard to its attempts to cope with its environment" and Stein et al. (2013)[4] uses the definition: "the quality of life as perceived by the animals themselves". The concept of the Five Freedoms (Table 1.1) are used widely in policy statements, legislation, and standards regarding humans' responsibility to care for animals and ensure good welfare[5–7]. However, this concept has been criticized for its focus on preventing negative states, rather than ensuring positive states; 4 of the 5 freedoms are focused on preventing negative states, such as thirst, hunger, malnutrition, discomfort, pain, injury, disease, fear, and distress[7,8]. Furthermore, the Five Freedoms provide guidance on management practices that merely keep animals alive, as opposed to providing an environment where the animals can thrive. So, animal welfare research has moved one step further, taking into consideration the assurance of positive emotions (or "positive welfare"), where the animal has "a life worth living"[7,8].

Table 1.1. The Five Freedoms and Provisions. Adapted from Brambell (1965)[9] and FAWC (1993)[10].

Freedom	Ensured by providing:
From thirst, hunger, and malnutrition	Ready access to fresh water and a diet to maintain full health and vigor
From discomfort and exposure	An appropriate environment, including shelter and a comfortable resting area
From pain, injury, and disease	Prevention or rapid diagnosis and treatment
From fear and distress	Ensuring conditions and treatment which avoid mental suffering
To express normal behavior	Sufficient space, proper facilities, and company of the animal's own kind

A more detailed conceptualization of animal welfare includes an interconnecting framework of 3 major components: affective states, biological functioning, and natural living (Figure 1.1)[11]. When considering a comprehensive approach to evaluating animal welfare, it is important to understand the complementary nature of these components and how they overlap. A focus on the assessment of one component can give an entirely different perspective on animal welfare than assessing animal welfare through either of the remaining two components[12]. The affective state viewpoint places emphasis on the animals' feelings and emotions (affective experiences), either positive, negative, or neutral. From this view, animals experiencing positive emotions, like pleasure and play, and free from negative emotions, such as pain or suffering, are considered to have good welfare. The second viewpoint, biological functioning, emphasizes aspects of good health, such as reproduction, nutrition, and growth. For example, good welfare from this standpoint would ensure that an animal that has an acceptable body condition score. Lastly, natural living places importance on the ability for the animal to live as it would in the wild. Good welfare would allow an animal to live in an environment that promotes natural behavior. In order to provide a complete evaluation of animal welfare, all three of these conceptions can be applied.

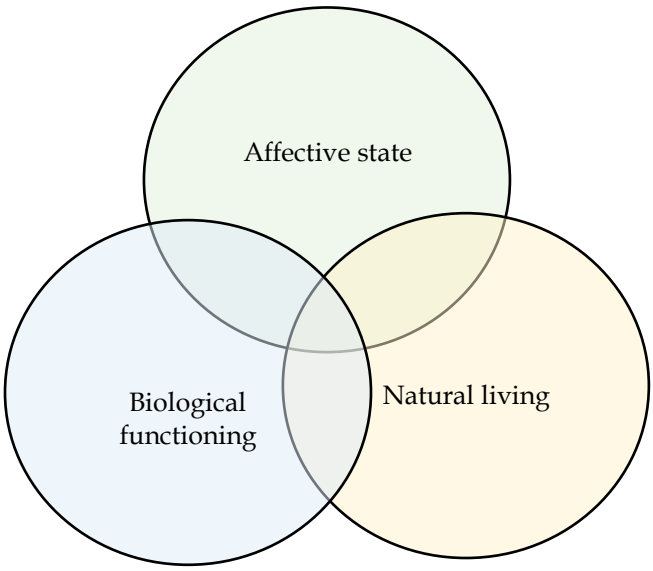

Figure 1.1. Three conceptions of animal welfare. Adapted from Fraser (2008)[11].

Emotions are a result of present situations that are potentially rewarding, such as access to food or mates, or punishing, such as threat from a predator, and everything in between[13]. Affective states occur in the absence of particular stimuli and are a cumulative product of past life experiences[13]. Thus, an animal is emotionally adapted to respond to its environment and this plays a large role in shaping that animal's affective state[13,14]. That is, if an animal is in an environment where it consistently experiences distress and lacks the opportunity to engage in preferred activities (leading to negative emotions), it will be in an overall negative affective state. On the contrary, an animal living in an environment that allows for expression of highly-motivated, species-specific behaviors (leading to positive emotions) will be in an overall positive affective state[13]. So, assessing affective state is a key component for evaluating animal welfare, as this can provide information on how an animal perceives its environment.

In the United States, the value of broiler chickens was $28.3 billion in 2019, with a total of 9.18 billion broilers produced[15]. The amount of chicken meat available per person has doubled since 1970 and as of 2018, chicken was the most widely available meat for consumption[16]. These data indicate an increase in the popularity and production of chicken, warranting investigation of their welfare and current housing systems. In 2020, the value of rainbow trout in the United States was $231.6 million, with a total of 149.6 million trout produced for sales or redistribution[17]. Even though trout are produced in substantial numbers, research on factors influencing their affective states are lacking compared to terrestrial agricultural species. Broiler chickens and rainbow trout are typically housed in barren environments at high stocking densities which can contribute to a lack of opportunity to express highly-motivated behaviors, potentially negatively impacting their affective states and ultimately their welfare. Therefore, we aimed to investigate how complex environments, in conjunction with varying stocking densities, influences affective states of broiler chickens and rainbow trout.

This thesis evaluates our current understanding of the factors that influence affective states of broiler chickens and rainbow trout. The following literature review (Chapter 2) focuses on current housing systems of broiler chickens and rainbow trout and their associated welfare concerns, environmental complexity, affective states, and cognitive

bias tests as reviewed in the current literature. Thereafter, three chapters are included, which have been submitted for publication in peer-reviewed journals. All three chapters focus on the impact of environmental complexity and stocking density on different aspects of broiler chicken and rainbow trout affective states. Chapter 3 investigates affective states of broiler chickens through a judgement bias test and Chapter 4 through attention bias and tonic immobility tests. Chapter 5 evaluates affective states of rainbow trout through a judgement bias test. Finally, a general discussion and conclusion are included in Chapter 7.

Chapter 2. Literature review

2.1 Broiler chickens

Broilers, a meat-type chicken, have been genetically selected for fast growth rates to produce meat for human consumption as efficiently as possible[18]. Over the past 50 years, slaughter age of broilers has decreased and market weight has increased[19]. On average, modern broilers are raised for approximately 47 days and reach a live weight of 2.90kg[19]. Two decades ago, broilers achieved a live weight of 2.3kg during the same 47 day rearing period, indicating an increased growth rate of 12%[19].

2.1.1 Environmental conditions

Under commercial conditions, broilers are raised in large, warehouse-like facilities at stocking densities of approximately 33-42kg/m^2 with access to feeders, water lines, and bedding material (e.g. wood shavings)[20]. Poultry houses are typically either 20 by 183m (66 by 600ft) or 13 by 155m (43 by 510ft) and can hold approximately 47,000 or 24,000 birds, respectively[21]. Modern poultry houses can be environmentally controlled through technology that maintains optimal temperature and ventilation[20]. According to the National Chicken Council (NCC) Animal Welfare Guidelines and Audit Checklist for broilers, lighting programs are especially important for "reducing behavioral problems, controlling growth, and improving musculoskeletal development" and broiler welfare is reduced when continuous lighting programs are used[22]. Lighting programs are dependent upon facility design, where some provide natural light through an open-sided house and others provide artificial light. The NCC Animal Welfare Guidelines and Audit Checklist requires 4 hours of darkness every 24 hours, except during the first and last week of growout[22].

Stocking density can be expressed as the total live weight of chickens present in a house at one time per square meter of usable area[23]. In order to maximize return on their investment and produce the most meat per house, broiler chicken producers will raise a

large number of broilers in a single house, resulting in a high stocking density[24,25]. Stocking density is typically determined by using target final weights and available floor space, adjusting for mortality, and calculating initial placement numbers so as not to exceed stocking density requirements[22]. Standards of practice can vary across the world. The United Kingdom Code of Practice does not allow stocking density to exceed 33kg/m^2, with exceptions allowing densities to reach 39kg/m^2 if certain requirements are met, such as detailed documentation of the production system[23]. Legislation according to the European Council Directive (2007/43/EC) permits stocking densities of up to 33kg/m^2, with exceptions to increase densities up to 42kg/m^2 for producers meeting certain requirements, such as documentation of good management practices and low mortality rates. In the United States, the NCC Animal Welfare Guidelines and Audit Checklist recommends a maximum stocking density of 36.6kg/m^2 (7.5lbs/ft^2) for broilers weighing 2 to 2.5kg (4.5 to 5.5lbs) and 41.5kg/m^2 (8.5lbs/ft^2) for broilers weighing 2.5 to 3.4kg (5.6 to 7.5lbs)[22]. There is no legislation on stocking density limits for commercial producers in the United States, however, 95% of chickens produced in the United States are raised by companies who are members of the National Chicken Council and adhere to their guidelines[26].

2.1.2 Broiler welfare concerns

There are a variety of welfare concerns associated with conventional broiler production, one of which is lameness (Figure 2.1)[27,28]. Lameness is a term interchangeable with leg weakness, both of which refer to "the group of pathologies which result in impaired walking ability"[28]. Broilers' genetic selection for high growth rate results in low activity levels due to the increased weight placed on developing bones and joints, and this consequence of growth rate is considered to be the main cause of lameness[29–31]. However, some previous studies report lameness to be associated with stocking density, disease, nutrition, air quality, light, age, body weight, and management practices[28,32–34]. For instance, broilers housed under a continuous lighting program showed an increased prevalence of impaired walking ability compared to broilers housed with an 8 hour dark

period every 24 hours[32]. A review of the aetiology and pathology of leg weakness in broilers concluded that although a multitude of factors, which are not mutually exclusive, contribute to leg weakness, the major contributing factor is genetic selection for high growth rate within a short life-span[28]. A study investigating the walking ability of 51,000 broilers found that over 27.6% of broilers ranging from 28 to 56 days old had gait scores consistent with poor locomotion and 3.3% were almost unable to walk[30]. Modern broilers spend significantly more time resting (53-86%)[35–40] compared to their wild ancestor (red junglefowl), who spend only 10% of their time resting[41], possibly because broilers can experience pain associated with locomotion[40,42]. Broilers sleep and lay down more when suffering from lameness[43]. These reduced activity levels lead to prolonged contact with (wet) litter, therefore increasing the risk of contact dermatitis[31,44]. Contact dermatitis (including hock burns, breast burns, skin lesions, or footpad dermatitis) results from prolonged contact of the body with wet litter and causes thickening or death of the tissue in the affected area[27,31,45]. Broilers with severe contact dermatitis exhibited slower weight gain, and researchers concluded this was a result of "pain-induced inappetence"[31,46].

Broiler welfare is jeopardized when stocking densities are over 34 to 38kg/m^2, which can contribute to reduced walking ability, impaired leg health, increased disturbances of resting birds, carcass bruising and scratches, and increased mortality[47]. Leg health and walking ability have been described as good estimators of poultry welfare[32,47]. When birds are housed at high densities, the length of walking bouts decrease as birds age[48], distance travelled decreases (42-46kg/m^2 compared to 30-34kg/m^2)[49], leg problems are worsened (40kg/m^2 compared to 34kg/m^2)[50], and the severity of contact dermatitis increases (34-36kg/m^2 compared to 32kg/m^2)[33] when compared to lower stocking densities. Broilers housed at high densities showed an increased occurrence of contact dermatitis[52–54], which were correlated with poor walking ability[52]. High stocking densities contribute to poor leg health and walking ability, however, environmental factors related to litter quality, lighting, temperature, and humidity can also impact leg health[31,44,47,55]. For example, there was an increased occurrence of hock lesions and leg deformities in broilers reared on wet litter[55]. Therefore, it is likely that leg health is a result of both stocking density and environmental conditions. Quality rest is important

for many physiological and maintenance processes, such as energy conservation, tissue repair and growth, and learning ability[56]. Rest is dependent upon lighting, temperature, length of dark period, and disturbances[56]. Interruptions of resting periods caused by disruptions from other birds can cause injuries, pain, and carcass damage from scratches on the disturbed bird[47]. In addition, repeated disturbances have been shown to cause distress[57]. When broilers are housed at higher densities, disturbances occur more often than at low densities[49,50,58]. At peak high density, broilers preferred to rest next to walls within the house, which the authors suggested was to avoid disturbances while resting[48].

Both a barren environment and high stocking density can exacerbate the aforementioned welfare concerns, as well as lead to limitation of behavioral expression through decreased activity levels[31]. Due to lack of complexity in the poultry house and limited available space due to high stocking densities, broilers are not provided the opportunity to engage in highly-motivated, species-specific behaviors important for ensuring good welfare and avoiding boredom- or frustration-related behaviors[59,60]. Three of these highly-motivated behaviors have been identified for broiler chickens: perching, dustbathing, and foraging[60–64]. As a natural behavior of red junglefowl (broilers' wild ancestor), perching on elevated surfaces is considered an anti-predatory behavior and provides protection while resting[56,65]. Perching has been suggested to improve leg health and mobility in broilers through increased movement[66]. When given easily accessible perches, broilers will exhibit perching behavior, suggesting they have maintained the motivation to perform this behavior[67]. Dustbathing is thought to "remove external parasites and improve feather condition" when birds repeatedly rotate and rub their bodies within a substrate, flap their wings, and stretch their legs[68,69]. Movements performed during dustbathing can help improve leg health and walking ability, and are considered to be exercise[68,69]. Dustbathing behavior is highly-motivated, as junglefowl deprived of substrate to dustbathe in will show compensatory dustbathing behavior when eventually provided with a substrate[70]. Junglefowl have also been observed attempting to dustbathe on wire floors, suggesting the motivation to perform this behavior is high, regardless of access to a suitable substrate[70]. Similarly, broilers will dustbathe throughout their life, and the motivation to dustbathe is so strong that they

perform the behavior even in unpreferred substrates[71]. Broiler chicks without leg impairments dustbathed every day, and those deprived of the opportunity to dustbathe exhibited increased dustbathing behavior once provided with dust[72], similar to laying hens and red junglefowl[43,70]. Foraging includes scratching and pecking at the ground, is considered an exploratory or food-seeking behavior, and occurs with or without subsequent feeding[73]. Regularly fed semi-wild junglefowl were observed foraging for 61% of the time they were active and spent 35% of their time scratching, which is a behavior associated with foraging[41]. Foraging is highly-motivated in domestic fowl, as they have been observed foraging in feces when not given a suitable substrate[74,75]. Broiler breeders are motivated to perform foraging behavior even when it does not fulfill their need to feed[73]. Commercial housing does not provide the opportunity for broilers to engage in these highly-motivated, species-specific behaviors, as they do not have access to perches or preferred substrate to dustbathe and forage in. Broilers do have access to litter, but this is typically reused between flocks. As birds age, litter quality can become suboptimal as moisture levels tend to increase, limiting the appeal for litter as a dustbathing substrate, which can contribute to the inhibition of dustbathing behavior[75–80].

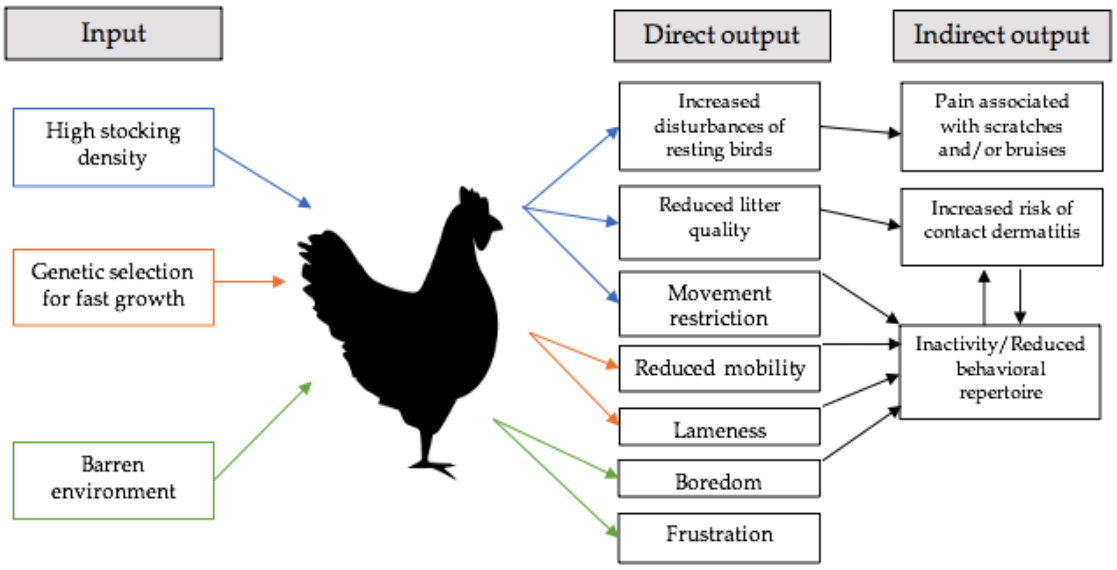

Figure 2.1. Summary of the identified major welfare concerns for broiler chickens and their consequences. Adapted from EFSA (2010)[27].

2.2 Rainbow trout

Rainbow trout are native to the Pacific Ocean tributaries in North America, but have expanded to occupy waters surrounding all continents except for Antarctica[81,82]. Strains can range from the anadromous steelhead trout, which reside in the ocean and move to rivers or streams to spawn (reproduce), to rainbow trout, which permanently reside in freshwater lakes[81–83]. In aquaculture systems, rainbow trout do not naturally spawn[82,84]. Therefore, eggs are artificially spawned from a mature broodstock[82]. Eggs extracted from female trout are mixed with sperm from multiple male fish (milt) and supplemented with water to facilitate fertilization[82]. While incubating, the eggs are left undisrupted in hatching troughs, incubators, or hatching jars[82]. Time until hatching is highly variable and dependent upon water temperature (e.g. 100 days at 3.9°C vs 21 days at 14.4°C)[82]. Once hatched, young trout are referred to as yolk-sac fry or alevins, due to their retention of the yolk sac (food reserve) for around 2-4 weeks post-hatch[82,84]. Alevins are housed in shallow troughs with little water flow during the yolk-retention life stage[82]. Once the yolk sac is absorbed, swim-up fry (previously alevins) develop food-seeking behavior and are moved to larger systems with increased water flow and depth[82,84]. Examples of these rearing systems may be circular tanks, either fiberglass or concrete, which ensures consistent water flow throughout the tank[82,84]. While the design of rearing systems can vary, circular tanks usually are 2m in diameter and 50-60m in depth[82]. During rearing, fry are typically hand-fed to avoid overfeeding and maintain uniform growth[82]. As fry continue to grow, temperature and dissolved oxygen levels are closely monitored, and fry are transported to even larger systems to reduce stocking density[82].

2.2.1 Environmental conditions

When young rainbow trout (fry) reach a length of 8-10cm and a weight of ~5g, they are moved to grow-out facilities[82]. There are 3 main systems for housing rainbow trout, each with potential risks for welfare: freshwater cages, flow-through outdoor raceways, and recirculating aquaculture tank systems[82]. Cage culture systems (in ponds or lakes) are typically 6m by 6m and 4-5m deep[82]. One advantage of freshwater cages over other,

land-based systems is that they utilize natural water currents, however, when water quality is suboptimal, it can be challenging to improve water conditions. Additionally, fish growth is dependent upon ambient water temperatures[82]. Good welfare is ensured through continual monitoring of fish behavior and condition. When rearing trout in freshwater cages, it can be difficult to identify and treat fish showing signs of reduced welfare. Another housing system is an outdoor raceway, which are usually 2-3m wide, 12-30m long, and 1-1.2m deep[82]. Water quality is maintained by a flow-through system, where water passes through once, then wastes are carried out of the system to be discarded[1]. However, fish are susceptible to external temperatures that can alter other water quality parameters, such as pH, and exposure to parasites or disease, potentially reducing fish welfare[82]. Lastly, recirculating systems are a relatively recent advancement in the aquaculture industry, where trout are reared indoors in environmentally-controlled conditions[85]. In contrast to flow-through outdoor systems, water is recirculated through a tank system and bio-filtered to maintain optimal water quality[86]. This system allows for year-round, large-scale trout production, however, a major drawback is that the system's components are connected[86]. During power outages, this can result in a total loss of fish if the grower does not respond fast enough[86]. Even if the grower does correct the problem within a reasonable timeframe, fish can still experience reduced growth and increased susceptibility to disease due to low oxygen exposure[86]. Trout are reared in these housing systems for around 9 months when they reach a market size of 30-40cm, and during this time, fish are frequently assessed for population uniformity (size)[82,86]. Fish graded out are either sold to alternative markets or euthanized[86].

A key component and potential stressor for trout production is fish stocking density. Determining a specific stocking density range is complex and requires consideration of fish age, water quality, social behavior, feed management, and type of rearing system[1]. In a production setting, stocking densities are usually determined based on prior knowledge of the system and species, with guidance from certain codes of practice, handbooks, and standards[87]. The Royal Society for the Prevention of Cruelty to Animals (RSPCA) developed a voluntary certification program based on welfare standards for the production of rainbow trout, and recommends that stocking densities not exceed

60kg/m^3 [88]. Another independent, third-party certification organization, the Aquaculture Stewardship Council (ASC), developed welfare standards for the production of freshwater trout and only requires that the maximum stocking density is determined with a designated veterinarian, without mention of specific stocking density ranges[89]. The Scientific Panel for Animal Health and Welfare (AHAW) of the European Food Safety Authority (EFSA) reports previously recorded stocking density ranges for the different rearing systems: 15-40kg/m^3 for cages, 150kg/m^3 for outdoor raceways, 25-45kg/m^3 for outdoor ponds, and 50-150kg/m^3 for recirculating aquaculture systems[86]. However, the EFSA also writes "where stocking density is relevant to welfare it is mediated through other variables such as water quality and fish behavior, meaning that stocking density per se is seldom a good way to predict welfare. Consequently it is difficult to set clear guidelines for both maximum and minimum stocking densities that would safeguard welfare"[86]. Ellis et al. (2002)[87] also noted the variation in stocking densities for different rearing systems: 4-55kg/m^3 for cages, 40-267kg/m^3 for tanks, and 8-160kg/m^3 for raceways. For this reason, the EFSA regards monitoring fish behavior and condition as the preferred method to evaluate their welfare[86].

2.2.2 Rainbow trout welfare concerns

A variety of welfare concerns for rainbow trout associated with high densities have been identified, such as altered swimming behavior, fin damage associated with aggression, and reduced water quality and feed availability (Figure 2.2). Swimming activity typically follows a diurnal rhythm[90–92]. This natural behavior can be disrupted when trout are reared at too high of densities for a certain housing system, as increased swimming activity or dispersed swimming has been linked with stressors like crowding[93]. When rainbow trout were housed at low stocking densities below 80kg/m^3, they exhibited circular swimming patterns during the day and reduced activity at night, indicating their natural swimming pattern was unaltered at this density[92]. However, rainbow trout housed at a density of 136kg/m^3 exhibited a high amount of nocturnal swimming behavior, similar to what was observed during the day. This suggests a lack

of suitable resting space and possibly exposes fish to distress because of the deviation from the natural diurnal swimming rhythm that was observed at lower densities[92].

Fin damage is a welfare concern for two main reasons: 1) fin tissue is living and contains nociceptors, therefore, damage to the fin can cause pain and 2) this damage can increase the risk for infection and disease[94–97]. A number of factors can cause fin damage, such as abrasion with tank walls, certain water quality parameters, or aggressive biting[87,98]. When trout are confined at high densities within a small area, their natural tendency for territorial defense is replaced by establishing a dominance hierarchy[99–102]. During the establishment of a dominance hierarchy, aggression is frequent and often results in nips or bites between individuals, with subordinate fish suffering from worse fin damage than dominant fish[103,104]. Rainbow trout housed at 10kg/m^3 showed better fin condition than fish housed at either 40 or 80kg/m^3, indicating fin damage increases with increasing density due to a greater chance for aggressive or accidental biting[105–107]. If abrasion with the tank walls also contributes to fin damage, then high density can be the factor that effectively worsens fin damage if optimal water quality is not maintained, as poor water quality can facilitate more rapid spread of pathogens[87,105,106].

High fish densities have been associated with reduced water quality and feed availability[108–110]. Fish are constantly in direct contact with their environment. Therefore, water quality greatly contributes to their physiological needs and overall welfare. Many water quality parameters must be kept within specific optimal ranges, depending on the species, including: dissolved oxygen, temperature, carbon dioxide, pH, ammonia, nitrite, and nitrate. Dissolved oxygen is important for the removal of metabolic wastes from water and serves as the medium for fish respiration[87]. As density increases, a greater number of fish are depleting the dissolved oxygen supply in order to respire, thus reducing water quality and increasing the risk of local hypoxia or asphyxia[1,111]. Low dissolved oxygen levels can elevate plasma cortisol levels, as fish reared in low dissolved oxygen environments (e.g. 50-60% dissolved oxygen in Atlantic salmon[112]) showed higher plasma cortisol than those reared in normal water quality environments, suggesting that reduced water quality can induce distress[113]. Fish excrete ammonia in an ionized and unionized form, the latter being toxic to fish[86]. Exposure to high amounts of

unionized ammonia can increase the risk of disease, gill damage, erratic swimming, loss of equilibrium, and death[86,87]. Fish tolerance to ammonia toxicity decreases with lower dissolved oxygen levels, therefore the risk of low dissolved oxygen levels as a result of increased activity at higher densities could impact ammonia toxicity in fish[86,114]. Feed availability is reduced at high densities, partially due to the dominance hierarchies that determine feeding order within the group[115]. Dominant individuals higher up in the hierarchy have a greater feed intake than subordinates, resulting in size variation that further establishes this hierarchy[87]. The reduction in feed intake by subordinates could be exacerbated by their elevated stress levels which suppresses appetite[116]. A lower ranking in the dominance hierarchy is associated with chronic stress (higher circulating cortisol concentrations compared to dominant fish), resulting in reduced growth rate[117]. In fact, rainbow trout injected with cortisol were more likely to become subordinate to smaller trout compared to rainbow trout not receiving a cortisol injection[117]. The reduction in feed intake may not be a function of reduced availability of feed from the higher consumption of dominant individuals, but rather a mechanism to avoid intraspecific competition. This effect of stocking density on feeding behavior was investigated by Boujard et al. (2002)[110], where in Experiment 1, rainbow trout were housed at three densities (100, 300, or 500 fish) in 1m^3 tanks and fed either in excess or by a self-feeder. Feed efficiency, body composition, and nutrient retention and loss were unaffected by treatment, however, growth and feed intake were negatively affected by increasing density, with an increase with growth heterogeneity. A possible explanation could be that feed accessibility was reduced due to monopolization of feeding areas by dominant individuals. In Experiment 2, the authors aimed to make food more accessible by providing trout (100 or 500 fish/m^3) with either one or three self-feeders. They discovered that feed intake was lower and growth heterogeneity was higher for trout housed at high densities. Additionally, the self-feeders were not used equally–one self-feeder was used more than the remaining two. These results suggest that feed availability or accessibility does not reduce feed intake, however, avoiding competition with conspecifics at a higher density is the mechanism by which subordinate trout reduce their feed intake[110]. On the other hand, rearing trout at too low of densities can diminish their welfare[87,107]. In small groups, trout can also show aggression and elevated

cortisol levels, while data on this in commercial conditions is lacking[87,107]. Fish will show anticipatory behavior prior to feeding, which includes increased swimming activity and crowding near the feeding area[118]. Apparent food anticipatory behavior is regarded as a sign of good welfare and suggests unstressed fish[119]. Reduced food anticipatory behavior is indicative of a stressed fish, where coping with the stress of a stimulus or event is a greater priority than feeding[118]. Fish housed at too low of densities have shown poor anticipatory behavior, indicating reduced welfare[87,106]. However, this could be due to the lack of visibility underwater and an already low number of fish, limiting the rapid cascade effect of feeding behavior within the group[91,120,121].

While farmed trout are housed in a way that allows for social contact, their environment is otherwise barren. In their natural environment, rainbow trout of all ages have access to underwater coverage such as aquatic vegetation or woody debris, water of varying depths, substrates such as gravel or cobblestones, and overhead coverage in the form of vegetation or undercut banks[81]. These structures and substrates are important for a variety of natural behaviors. Overhead or underwater cover provides trout with shade, protection from predators, and a safe place to rest[81,122,123]. Additionally, dependent upon their individual activity (e.g. resting, feeding, foraging), trout show a preference for different water velocities that can be provided by underwater coverage and vegetation[81]. For example, when foraging for food, trout occupy areas with reduced water velocity next to fast-moving water, which dislodges food items[81,122]. Adult trout naturally establish territories, which are dependent upon the complexity of their habitat, predator density, and food availability[81]. In comparison to their stimuli-rich, complex, natural environment, conventional housing of farmed rainbow trout is barren and does not provide fish with the opportunity to show certain natural behaviors, such as finding protection under overhead coverage, establishing territories, or seeking preferred microhabitats. Within the dominance hierarchy established under artificial conditions, subordinate fish suffering from repeated attacks would have no coverage in which to seek protection[119]. This issue becomes increasingly concerning in fish reared for redistribution into the wild. Fish reared in barren environments show a reduced ability to perform behaviors essential for survival, such as feeding and avoiding predation, and are generally not as successful as their wild counterparts[124,125]. An open-access database,

which analyzed the literature on 41 aquaculture species and bibliographical reviews on the biology of wild and captive species, concluded that the welfare state of farmed fish, including rainbow trout, is poor with room for improvement[126].

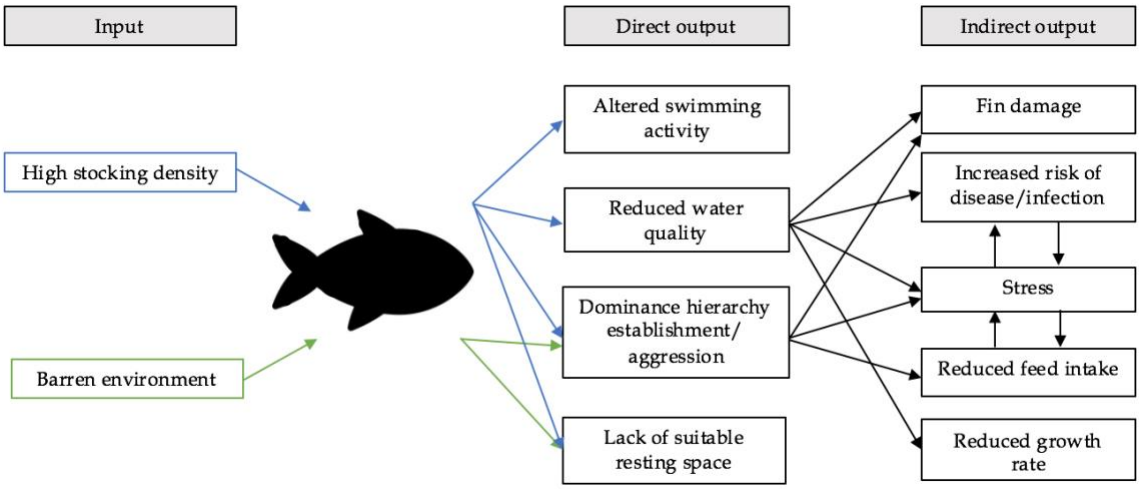

Figure 2.2. Summary of the identified major welfare concerns for rainbow trout and their consequences.

2.3 Environmental complexity

Environmental enrichment (complexity) can be defined as "a modification of the environment of captive animals, thereby increasing the animal's behavioral possibilities and leading to improvements of their biological function"[127]. Riber et al. (2018)[59] outlined the objectives of environmental enrichment, which are to:

1) increase the occurrence and range of the animal's normal or species-specific behavior

2) prevent the development of abnormal behavior or reducing its extent and complexity

3) increase the positive exploitation of the environment

4) increase the animal's ability to handle behavioral and physiological challenges

Van de Weerd and Day (2009)[128] proposed a framework built upon the former which involves the economic aspects of environmental enrichment. The authors define successful enrichments as meeting four criteria:

1) it should increase species-specific behavior

2) it should maintain or improve levels of health

3) it should improve the economics of the system

4) it should be practical to employ

In order to provide successful enrichments, it is important to understand a species' behavioral repertoire in their natural environment[129]. However, Newberry (1995)[127] suggests the functionality of behavior in a specific captive environment is more useful than the behavior's naturalness. Some behaviors performed in the wild are not beneficial for animals to perform in captivity[127]. For example, the natural anti-predator response to flee or hide might prevent a captive animal from receiving adequate and necessary medical care from caregivers. Therefore, the reason why animals perform certain highly-motivated behaviors should be considered when developing and implementing successful enrichments[127]. A functional target behavior should be identified and defined for the species, where enrichments are then utilized to promote that target behavior[128].

Taking this into consideration, environmental enrichment should increase an animal's opportunity to perform motivated behaviors functionally adaptive in a captive environment (promoting positive affective states and avoiding negative affective states) and improve their biological functioning[130]. Through increased complexity of the environment, animals are less likely to experience frustration- and boredom-related behaviors (negative valence with high and low arousal, respectively) which can negatively impact animal welfare and affective state[127]. For example, broiler breeders (parent flocks of broiler chickens) housed in a barren environment under feed restriction can show stereotypic object pecking and aggression[131]. The occurrence of these abnormal behaviors can be reduced by providing substrate, which would be considered a successful enrichment[132]. Furthermore, barren environments may not provide animals

with the opportunity to engage in highly-motivated behaviors, deprivation from which can cause frustration[127]. Animals prevented from feeding may exhibit frustration in the form of excessive pacing, aggression, or grooming behavior[127,133]. Preventing goal-directed behaviors that do not fulfill a specific physiological requirement (e.g. foraging behavior that does not reduce hunger) can be one cause of psychological suffering[134–136]. For instance, laying hens spend the same time foraging in litter, regardless of the presence of feed in the litter, indicating the need to forage regardless of the lack of a physiological reward in terms of reducing or avoiding hunger[137]. When hens had to pay a cost of squeezing through a narrow entrance to access resources (food and water, wood chips, grass or wheat seedlings, perch, nest box, or a pen facing another pen with familiar hens), time spent foraging did not differ compared to when they had free access to resources, suggesting a strong motivation to perform foraging behavior[138]. Dustbathing behavior, thought to clean plumage, was performed in a compensatory manner after prevention of dustbathing in both feathered and featherless domestic chicks[139]. By providing meaningful enrichments developed with a certain species or individual animal in mind, negative experiences (e.g., mental suffering from the inability to perform certain highly-motivated behaviors or aggression) can be minimized and positive experiences can be promoted. In other words, the bare minimum is preventing pain and suffering, but we should strive to provide animals with the opportunity to engage in preferred behaviors (through the use of enrichments), ultimately contributing to a positive affective state and positive welfare.

In addition to promoting positive, species-specific behaviors, enrichments should improve the biological functioning of animals. It is important to consider that if an enrichment has a negative impact on production or health, it is unlikely to be accepted into a large commercial setting[128]. By increasing activity levels through engaging in species-specific behaviors, it is likely that aspects of health and biological functioning are also improved as a result of providing environmental enrichment[59]. It is important to note that activity level should not be the sole indicator of welfare, rather behavioral diversity and engaging in positive activities[140]. For example, stereotypies such as pacing increase activity levels, but are generally regarded as an indicator of poor welfare due to increased time spent on one behavior and a subsequent lack of behavioral diversity[140].

Certain enrichments provide animals with the opportunity to engage in constructive activities that require movement, therefore improving their muscular, skeletal, and cardiovascular systems and reducing the risk of injury[59,127]. Other enrichments can reduce fear or stress levels, preventing panicked responses to environmental change or handling that might result in injuries[141]. Detrimental effects of a barren environment that prevent animals from performing certain behaviors can manifest as abnormal behaviors that cause injury and a reduction in biological functioning in other animals within the group. For example, rooting and foraging are natural behaviors of pigs and cannot be performed in a commercial setting due to lack of substrate[142]. Boredom or frustration due to this lack of stimulation in the environment is thought to be one cause for the abnormal behavior of tail biting, which is a major welfare concern as victims of tail biting can experience reduced biological functioning in the form of painful tail lesions, infection, and decreased growth[143,144]. Providing enrichments, such as straw to forage in or toys to explore, can reduce boredom or frustration, leading to a reduction in tail biting and improvement in biological functioning of the group[128,145,146].

In recent years, there have been some legislation changes in regards to laying hen welfare. For example, the European Union passed a directive in 2012 which banned the use of non-enriched caged systems (battery cages) for laying hens[147]. The directive also provides standards for rearing laying hens, in which different types of housing systems are described, such as enriched cages or alternative systems[147]. Both enriched cages and alternative systems require perching space, nests, and litter. In comparison, the Council Directive does not specifically mention the use of environmental enrichments to improve housing conditions for broiler chickens[148]. Some retailers in the United Kingdom, Netherlands, and Scandinavia raise broilers under "higher welfare indoor systems", which provide straw bales and/or perches, natural light, and lower maximum stocking densities[149]. While laying hen welfare legislation is in place in the European Union and some areas of the United States, broiler chickens and fish are under no welfare legislation that might improve housing conditions and animal welfare.

2.3.1 How environmental complexity can help resolve broiler welfare concerns

Environmental enrichment (complexity) has been suggested as a solution to the welfare concerns regarding conventional broiler production, with one example being the provision of perches (Table 2.1). While the use of perches varies in broilers dependent on genotype, age, stocking density, and environmental temperature, providing perches has been positively correlated with increased activity levels[59,150,151]. This increased activity level linked with providing perches has shown to improve leg health through reduced occurrence of tibial dyschondroplasia[152]. Broilers provided with natural light, wood shaving bales, perches, and metal chains were more active, observed through more walking, exploration, and foraging, compared to broilers housed without enrichments[153]. Additionally, access to perches can reduce hock burns and footpad dermatitis (erosions of the skin that can lead to discomfort, lameness, or secondary infection), while also improving plumage condition, possibly through reduced contact with litter material[154,155]. Perching allows broilers to escape contact with wet or warm litter, as broilers show a preference for cooler sections of a perch over warmer sections[154,156]. Disturbances of resting birds can be minimized by providing perches, as broilers resting on a perch will possibly avoid the activity of birds walking on the floor[59,157]. However, use of perches can be low and their beneficial effect minimized if the design does not consider broilers' fast growth rate and relatively weak bones[59]. For example, broilers have shown a preference for suspended, raised platforms rather than perches with bars observed through a higher rate of successful perching attempts, possibly because the platforms were easier to access and balance on[158]. Platforms are a different type of elevated surface that can be provided for broilers, which are typically raised, slatted areas that broilers can access by walking up a ramp[59]. Birds with access to platforms had lower mean gait scores and occurrence of tibial dyschondroplasia, correlating to better leg health, than those without access to platforms[66,150]. However, footpad condition or general activity level was not affected in those studies.

Straw bales or other substrates have been investigated as a source to stimulate activity and improve leg health in broilers, yet their effect on broiler distribution within the house is not known. A study investigating the effect of one straw bale per 17m^2 on the

behavior and activity of broilers discovered that the straw bales were used by the broilers to sit on or next to and overall, had increased activity levels compared to those housed without straw bales[159]. When one bale per 44m^2 was provided with natural light, broilers housed with straw bales had longer latencies to lie, suggesting better leg health and walking ability, than broilers housed without straw bales[160]. However, no differences were found between treatments on overall activity level or time spent sitting. When straw bales were provided at different densities, broilers housed with one bale/29m^2 showed longer latencies to lie compared to broilers housed with one bale/44m^2, however, there was no effect of bales on activity levels, lameness score, or contact dermatitis[161].

Enrichments aimed to stimulate foraging and dustbathing behavior have demonstrated that broilers prefer certain substrates. In preference tests, broilers provided with sand, wood shavings, rice hulls, recycled paper, and no litter spent the most time in sand and preferred to dustbathe in sand over other substrates[69,162]. When given both sand and wood shavings, broilers spent a greater proportion of their time on sand[71] and spent more time foraging compared to broilers with access to only wood shavings[163]. However, when given either sand or wood shavings, broilers exhibited a similar time budget, suggesting that broilers prefer sand when given the choice between two substrates, but behavior was not altered when given only one substrate[71]. Broilers given a choice between moss-peat, oat husks, straw pellets, or clean wood shavings preferred to dust bathe and forage in moss-peat and oat husks[76]. These studies demonstrate that broilers do have a substrate preference in which to perform certain behaviors, and that dustbathing activity may be stimulated when broilers are given a choice between at least two substrates.

Hanging strings have been investigated as a potential way to encourage natural behavior. Location and lighting play a role in string enrichment effectiveness. Broilers' interaction with the strings and pecking activity is low when provided with low light intensity[163]. Broilers given access to hanging strings near feed interacted with the strings more frequently at 3 weeks of age compared to weeks 4 and 5. Additionally, gait scores of broilers with access to strings were improved during weeks 3 and 4 compared to

broilers housed without strings[164]. These studies show the potential of providing string enrichments as a way to increase activity level through pecking behavior, however, their effect on leg health and walking ability is somewhat unclear.

Environmental enrichment is thought to reduce fear in poultry[165]. After exposure to acute stress, broilers housed with colored plastic balls, plastic bottles, and mirrors (rotated every 3 days) had shorter tonic immobility durations than broilers housed in a barren environment, suggesting birds housed with environmental enrichment were less fearful following acute stressors[141]. Some studies have shown a reduction in fearfulness of broilers housed with access to platforms compared to broilers housed without platforms[166,167]. However, other studies found no effect of environmental enrichment, including perches and barriers on fearfulness in broilers[155,168,169].

Environmental enrichment can benefit not only broilers, but also producers. Some commercial production systems incorporate the provision of perches, straw bales, natural light, lower maximum stocking densities[59]. The UK's RSPCA's Freedom Food scheme provides straw bales, perches, pecking objects, a lower stocking density ($30 kg/m^2$), and uses a slow-growing genotype. This higher welfare system was compared to conventional broiler production, which uses a fast-growing genotype and stocking density of $38 kg/m^2$. Broilers in the higher welfare systems showed lower mortality, less severe hock burns, and less severe footpad dermatitis compared to the conventional system[170]. Producing broilers in the higher welfare system costed more, but when the mortality and slaughterhouse losses were included, there were economic benefits to raising broilers under the higher welfare system[170].

Table 2.1. Summary of the effects of environmental enrichment on aspects of broiler chicken welfare. Adapted from Riber et al. (2018)[59]. + = improved, 0 = no effect, - = reduced, and ? = unclear.

Enrichment	Effect on						
	Activity	Leg/foot problems	Fear	Homogenous bird distribution	Disturbances	Growth	Heat stress
Perches	+[59,150,151]	-[152,154,155] or 0[171]	0[168,169]		-[157]	0[168,171,172] or +[173]	-[154,156]
Platforms	0[66,174] or +[175]	-[66,150,174]	-[166,167]				
Panels	-[176]	-[177]		+[176,178]	-[58]		
Barriers	0[157,179]	-[155,157]	?[155]	+[58]	?[155,157,176,178,180]	0[155,179]	
Straw bales	?[159–161]	-[160,161]					
Hanging strings	+[164]	-[164] or 0[51]				0[51]	
Novel objects			-[141]				

2.3.2 How environmental complexity can helps resolve rainbow trout welfare concerns

Environmental enrichments, like shelters, overhead cover, and substrates, can help improve fish welfare (Table 2.2). Shelters provide fish with a place to hide and reduce aggression[181]. Juvenile trout reared with submerged tree tops, overhead cover, and underwater feeders had better fin condition than trout reared in control tanks, suggesting a reduction in aggressive behavior[182]. Overhead coverage provides lower light intensities, which is preferred by salmonids[183]. When provided with plywood coverage of varying sizes (0.3 x 0.3m, 0.6 x 0.6m, or 0.9 x 0.9m), rainbow trout showed a preference for the largest overhead cover, with highest levels of activity outside of the shaded area, possibly because rainbow trout prefer to rest in shaded areas and be active

in the lighter areas[184]. Additionally, juvenile rainbow trout reared with partial or near full overhead cover had better weight gain and feed conversion ratio than trout reared without overhead cover[185,186]. Adding substrate and artificial plants to a tank can provide fish with a more natural environment and improve their health and welfare compared to rough tank bottoms used in commercial settings, such as concrete. Rainbow trout reared with cobblestone[187] or gravel[188] tank bottoms had better fin condition than trout reared in control conditions, which could be due to reduced abrasion with tank surface and reduced aggression. Juvenile rainbow trout housed with stones and plastic plants showed increased exploratory behavior compared to those housed without enrichments[189]. Furthermore, rainbow trout housed with gravel, plants, and overhead cover recovered quicker from an acute stressor (1 minute of air emersion), measured through latency to resume to normal behavior, than trout housed in a barren environment[190]. However, recovery rate did not differ between treatment groups following a painful stimulus (subcutaneous injection of acetic acid). These studies show the beneficial effects of environmental enrichment on aspects of rainbow trout welfare, such as better fin condition and improved growth.

Other forms of enrichment, like vertically-suspended aluminum rods, angles, or strings, have shown to improve fish performance. Juvenile rainbow trout reared with vertically-suspended strings attached with colored plastic spheres had better weight gain, feed conversion ratio, and individual fish length and weight than fish reared without enrichments[191]. Additionally, rainbow trout housed with one or two arrays of vertically-suspended aluminum angles were longer, heavier, and had a higher growth rate than trout housed without enrichments[192].

Table 2.2. Summary of the effects of environmental enrichment on aspects of rainbow trout welfare. + = improved, 0 = no effect, HT = hatchery tank, CT = circular tank, CR = concrete raceway, RT = recirculating tank (* = semi-recirculating tank).

Rearing system	Enrichment	Effect on							
		Fin condition	Weight gain	Feed conversion ratio	Condition factor	Final tank weight	Individual weight & length	Specific growth rate	Behavior
HT	Submerged tree tops, overhead cover, underwater feeders[182]	+							
CT	Partial overhead cover with block substrate[185]		+	+					
CT	Partial (65%) or near full (98%) cover[186]		+	+	0				
CR	Cobblestone bottom[187]	+							
CR	Gravel bottom[188]		+	+		+			
RT	Rocks, plastic plants (stable)[189]								+
RT*	Gravel, plastic plants, overhead cover[190]								
CT	Overhead cover with either vertically-suspended aluminum rods or colored balls[191]		+	+					
CT	1 or 2 arrays of vertically-suspended aluminum angles[192]		0	0			+	+	
CT	Near full cover with vertically-suspended aluminum rods[193]		+	+					
CT	Vertically-suspended aluminum rods or angles[194]		+	+					
CT	Vertically-suspended		+	+	0		0		

Rearing system	Enrichment	Effect on							
		Fin condition	Weight gain	Feed conversion ratio	Condition factor	Final tank weight	Individual weight & length	Specific growth rate	Behavior
	pieces of plastic conduit[195]								
CT	Vertically-suspended aluminum angles with exercise (5cm/s to 8cm/s or 11cm/s water velocity biweekly)[196]		0	0	0	0	0	0	
CT	Vertically-suspended aluminum angles or exercise (12.2cm/s to 30.5cm/s water velocity weekly)[197]		+	+ (angles only)		+		+	

2.4 Affective state: Defining relevant terms

While the terminology of "emotion," "mood," and "affective state" are widely reported throughout human and non-human animal research, their definitions vary in the scientific literature[198,199]. This has contributed to an uncertainty in how to measure, study, and interpret animal emotion and mood[200]. In fact, the disconnect between the use of these terms and what they mean has contributed to the argument on which species feel emotions and which are unable to do so[198]. Definitions of emotion can vary. Three definitions are:

"Certain neurophysiological states, inferred from behavior, about which little is known except that by definition they predispose toward certain specific kinds of action"[201]

"A temporary state brought about by biologically relevant external stimuli, marked by specific changes in the organism's body and mind"[202]

"Short-term states elicited by stimuli (or their predictors) that animals will work to acquire (rewards; e.g. prey) or avoid (punishments; e.g. predators)."[14]

These definitions of emotion are ordered in terms of increasing specificity. The first definition hesitantly approaches the term, implying emotion influences an animal's actions, but does not specify the nature of those actions or duration of the neurophysiological state. The second definition provides a more detailed explanation of emotions, which suggests they are generated by biologically-relevant external stimuli and produce distinct biological changes, also noting emotions' short-term nature. However, this definition neglects to account for outward behavioral expression of emotion. The last definition encompasses the most detailed explanation of emotions, including how they are produced, what occurs as a result, and their duration. Although each definition can vary in terminology, all definitions of animal emotion in scientific literature seem to have general themes, such that it is a biologically comprehensive (in terms of behavior, physiology, and subjectivity) and valanced (positive or negative) response to a stimulus within the environment that can differ in arousal and duration[203]. In this thesis, we choose a definition of emotion slightly modified from Crump et al. (2020): short-term functional states produced by stimuli that animals work to either gain (reward) or avoid (punishment), which help the animal to appropriately respond to environmental changes[14].

On the other hand, mood has been described as an ongoing state that an animal experiences without the presence of a certain stimulus, event, or object[204]. More specifically, it has been defined as "the outcome of the accumulation of short-term emotional experiences, resulting in a 'running mean' of positions occupied across scales of valence and arousal over time"[13,205,206]. Keeping in mind our definition of emotion, which is partially characterized as a short-term state, mood is defined as a long-term, diffuse state that reflects the cumulative valence (positive or negative) of emotions over time[13]. In other words, the valence of short-term emotions an animal experiences throughout life will determine that animal's mood. Affective state and mood have been used interchangeably[14]. But affective states have also been described as a combination of an animal's long-term mood state, as well as the animal's short-term emotional response

to current events, such that an animal in a negative long-term mood state may still temporarily experience positive affective states as a result of a reward (e.g. food or mating success)[13]. In this thesis, we approach the definition of affective state as an umbrella term for mood, as it is generally a more technical rather than conversational term[200]. By using affective state instead of mood, we can avoid an anthropomorphic interpretation of these long-term valanced states[200].

Valence and arousal are the two core factors considered to conceptualize affective state[13,204]. Valence refers to how an animal perceives a situation, ranging between positive (pleasant, rewarding) and negative (unpleasant, punishing)[14,200]. Whereas arousal is the intensity of an emotion and determines how vigorously an animal responds to a stimulus[200]. The interaction of valence and arousal are shown in Figure 2.3, where Q1 and Q2 make up positive affective states and Q3 and Q4 are characteristic of negative affective states[13]. When looking at each quadrant separately, Q1 represents a high arousal, positively valanced state (e.g. "excited" or "happy")[13,14]. Animals in this positive, high arousal affective state generally exhibit motivational behavior, in which they actively work towards acquiring a reward[13,207–209]. For example, a predator that has successfully spotted and captured its prey, leading to a state of excitement. In contrast, negative, high arousal affective states in Q4 are associated with "fear" and the appropriate behavioral responses to threatening or dangerous stimuli[13,207,208]. An animal in this state may have encountered a predator in which immediate action was taken to avoid predation. Negative, low arousal affective states in Q3 can be described with the terms "sadness" or "depression" and are reflective of an animal that has lost a reward or is in an environment in which resources are limited[13,210]. Lastly, Q2 represents a low arousal, positively valanced state, associated with "calm" or "relaxed" feelings. An animal in this state is usually in an environment where its needs are met with little threatening or dangerous stimuli[13,207].

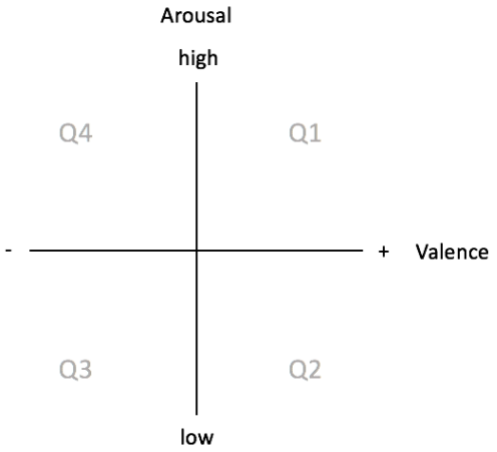

Figure 2.3. Depiction of how valence and arousal make up affective states (Q1, Q2, Q3, and Q4). Adapted from Mendl et al. (2010)[13] and Crump et al. (2020)[14].

2.5 Quantifying affective state: Cognitive bias tests

Cognition is defined as the mechanism by which animals acquire, process, store, and act on information from their environment[211]. Through overlapping brain regions, affective states influence cognitive processing and cognitive processing influences affective states[212–215]. In humans, affective state influences aspects of cognitive processing, such as decision-making[13,216–218]. In this manner, humans in negative affective states tend to judge ambiguous stimuli negatively[219], are more likely to focus on threatening stimuli, and more readily remember negative memories than humans in positive affective states[220,221]. As an example, an animal in a negative affective state resulting from a highly threatening environment will process ambiguous stimuli (a rustle in the grass) as a negative situation (predator), remember what has happened in similar past situations, and take action to seek safety[13]. In the same situation, an animal in a positive affective state resulting from a comfortable environment with plentiful resources might interpret the same ambiguous stimuli (rustle in the grass) as a positive situation (food source or potential mate), remember what has happened in similar past situations, and act to obtain the food reward or attract a potential mate. As such, there is an evolutionary benefit of the co-dependence of emotion and cognition for animals which has been favored by selective pressures[222,223]. When an animal's affective state impacts aspects of

cognition, such as judgment, attention, and memory, it is called a "cognitive bias"[215,223–225]. Therefore, cognitive biases can be used as an indicator of animal affective state and welfare by providing information on how the animal perceives their environment[13,207,211,215,226–228].

There are several advantages to using cognitive bias tests to assess animal affective state and welfare. A majority of animal welfare studies evaluate negative states due to the ease of measuring the subsequent physiological and behavioral responses compared to those associated with positive affective states, and the greater priority placed on the avoidance of suffering[223,229]. However, evaluation of good welfare involves not only the prevention of negative states, such as stress and pain, but also the assurance of positive states, elicited by behaviors such as exploration and play[136,230,231]. Traditional behavioral measures of animal welfare can be interpreted subjectively, as verbal communication of an animal's needs is not possible, and may be hard to determine due to species differences[224]. For example, how might we differentiate a resting animal who's basic needs are met from an animal resting due to depression or boredom[227]? Therefore, the utility of cognitive bias testing is that it allows for objective evaluation of positive affective state in animals[227]. Furthermore, conducting cognitive bias tests allows for differentiation between affective states and arousal[227]. Physiological measures of animal welfare, namely corticosteroids, can indicate an increase in arousal resulting from an ambiguous situation, however the conclusion that this spike in arousal is due to either negatively (stress) or positively (excitement) valanced emotions cannot be made, unless the animal is in a clearly negatively valanced situation (e.g. rough handling)[13]. An example of this might be feed anticipation, which elicits high arousal and can be deemed positively valanced through subsequent consumption or negatively valanced through frustration if the feed is withheld for a prolonged period[232,233]. Additionally, cognitive bias tests allow for *a priori* hypotheses following manipulation of affective state, for example, through altering housing conditions[227]. This ultimately allows for effective evaluation of management practices, environmental enrichments, and other interventions on affective state. Three types of cognitive biases exist: judgement, attention, and memory[223]. Here, we focus on judgement and attention biases.

2.5.1 Judgement bias tests

Judgement biases refer to an individual's interpretation of ambiguous events[215]. More specifically, we attempt to answer the question "does the individual predict a positive or negative outcome from this ambiguous event?". The nature of this anticipation can be defined as either 'optimistic' or 'pessimistic'[215,234]. Individuals who are in a positive affective state tend to interpret ambiguous stimuli more optimistically than those in negative affective states, who would interpret the same stimuli pessimistically[222]. Therefore, judgement bias testing is used to determine levels of optimism and pessimism of individuals based on their responses to ambiguous cues during testing. In fact, a meta-analysis of 71 judgement bias studies on 22 species showed optimistic and pessimistic responses to ambiguous situations resulting from positive or negative affective states, respectively[235]. Judgement bias cues can be spatial[236], visual[237], auditory[238], olfactory[239], tactile[240], or a combination of these (multimodal)[227]. Although judgement bias tests are applied to evaluate affective states of a wide range of species, there is currently no evaluation of this in rainbow trout and one published study for broiler chickens.

Three types of judgement bias tasks described in the literature are:

- Go/No-Go
- Active choice (Go/Go +) with positive reinforcement
- Active choice (Go/Go -) with negative reinforcement

In the Go/No-Go judgement bias task, spatial cues are widely used, where subjects are trained to approach one location to receive a reward (e.g. food) and avoid approaching another location which would result in an aversive stimulus or no reward (e.g. unpalatable food or no food at all)[227]. After successful discrimination between the two reinforced locations, responses to ambiguous cues at intermediate locations are recorded as latencies to approach. Animals in a positive affective state are expected to show

shorter latencies to approach a wide range of ambiguous cues, suggesting the animal anticipates a positive outcome in novel situations, indicative of an optimistic response. Alternatively, animals in a negative affective state are expected to show longer latencies to approach a narrower range of ambiguous cues, suggesting the animal anticipates a negative outcome in the same novel situation and is indicative of pessimism[222,223,235,241]. Affect manipulation impacting responses in the Go/No-Go judgement bias task have been demonstrated in dogs[236,242–245], pigs[246,247], goats[248], cattle[249,250], sheep[251–261], horses[262], cats[263], primates[264,265], mice[239,266], rats[267–270], starlings[271,272], chickens[237,241,273–275], and fish[276–278]. However, this type of task has been criticized for the unclear motivation behind the "No-Go" response. In other words, the lack of approach could be due to the learned discrimination between "Go" and "No-Go", but could also be due to lack of motivation, confusion, or distraction[211,215,279].

In order to mitigate this issue, the active choice task was developed using positive reinforcement, where the animal is required to make an active response to both cues[211,227]. In this type of task, subjects are trained to discriminate between a cue that results in a high reward and another cue that results in a low reward. While not as widely used as Go/No-Go tasks, impacts of affective state on judgement biases in active choice tasks have been found in pigs and rats using auditory signals[280,281], as well as grizzly bears, starlings, and capuchin monkeys using visual signals[234,282,283]. One issue with the active choice task is that the dual-reward aspect could limit our understanding of judgement biases regarding neutral or negative situations[222]. This concern led to the development of an active choice task involving negative reinforcement. In other words, animals are trained to respond to a stimulus to obtain a reward and respond to another stimulus in order to avoid a punishment. This has previously been demonstrated, where rats were trained to press a lever located on the left side of a feeder to receive a reward (sucrose solution) and to press a lever located on the right side of a feeder to avoid a punishment (electric shock)[284]. While this type of judgement bias task may raise ethical concerns for animal welfare research due to the involvement of negative reinforcement, results from studies utilizing this task are in alignment with those of Go/No-Go and active choice task [227]. Studies evaluating affective states of livestock species using a judgement bias test are summarized in Table 2.3.

Table 2.3. Summary of studies evaluating affective state of livestock species through a judgement bias test. Adapted from Bethel (2015)[227].

Species	Test type	Cue	Response	Affect manipulation	N tested	Result	Reference
Goat	Go/No-Go	Spatial	Approach	N: poor welfare C: good welfare	18	Females from N more optimistic	Briefer & McElligott (2013)[248]
	Go/No-Go	Spatial	Approach	P: gentle grooming C: no grooming	19	No effect	Baciadonna et al. (2016)[285]
Dairy calf	Go/No-Go	Visual	Approach	N: separation from dam C: with dam	13	N calves more pessimistic	Daros et al. (2014)[249]
	Go/No-Go	Visual	Nose touch	N: after hot-iron disbudding C: before hot-iron disbudding	17	N calves more pessimistic	Neave et al. (2013)[250]
Pig	Go/No-Go	Spatial	Approach	N: social isolation C: normal housing	32	No effect	Düpjan et al. (2013)[286]
	Go/No-Go	Spatial	Approach	N: high density C: low density	40	No effect	Scollo et al. (2014)[246]
	Go/No-Go	Auditory	Approach	P: enriched → barren → enriched housing N: barren → enriched → barren housing	10	Both groups optimistic in current enriched housing Pigs moved from enriched to barren housing more pessimistic	Douglas et al. (2012)[247]
	Active choice (+)	Spatial & auditory	Approach	N: restraint C: no restraint	15	No effect	Murphy et al. (2013)[238]
	Active choice (+)	Auditory	Approach	N: low birth weight C: normal birth weight	16	N less optimistic	Murphy et al. (2015)[280]

Species	Test type	Cue	Response	Affect manipulation	N tested	Result	Reference
Sheep	Go/No-Go	Spatial	Approach	N: unpredictable environment P: unpredictable → positive environment	30	P more optimistic	Destrez et al. (2014)[254]
	Go/No-Go	Spatial	Approach	P: treated with diazepam C: control	16	P more optimistic	Destrez et al. (2012)[251]
	Go/No-Go	Spatial	Approach	N: unpredictable, aversive events C: control	48	N more pessimistic	Destrez et al. (2013)[252]
	Go/No-Go	Spatial	Approach	N: aversive events C: control	26	N more pessimistic	Doyle et al. (2011)[255]
	Go/No-Go	Spatial	Approach	N: unpredictable, stimulus-poor housing P: predictable, stimulus-rich housing	24	N slightly more optimistic	Guldimann et al. (2015)[259]
	Go/No-Go	Spatial	Approach	N: restraint and isolation stress C: control	20	N more optimistic	Doyle et al. (2010)[258]
	Go/No-Go	Spatial	Approach	N: depletion of brain serotonin (administered pCPA) C: water	30	N more pessimistic	Doyle et al. (2011)[256]
	Go/No-Go	Spatial	Approach	Test effect of repeated testing	15	Sheep learned ambiguous cues were unrewarded	Doyle et al. (2010)[257]
	Go/No-Go	Spatial	Approach	N: short-term stress of shearing C: no shearing	24	N more optimistic	Sanger et al. (2011)[260]

Species	Test type	Cue	Response	Affect manipulation	N tested	Result	Reference
	Go/No-Go	Spatial & visual	Approach	N: restraint and isolation stress C: control	30	N slightly more optimistic	Verbeek et al. (2019)[287]
Laying hens	Go/No-Go	Spatial	Approach	N: basic pens P: enriched pens	27	No effect	Wichman et al. (2012)[288]
	Active choice (+)	Spatial & visual	Approach	N: 5-min social isolation C: control	20	No effect	Hernandez et al. (2015)[289]
	Go/No-Go	Visual	Peck	N: cold environment P: hot environment	5	P more optimistic	Deakin et al. (2016)[241]
	Go/No-Go	Spatial	Approach	N1: chicks denied access to litter area N2: chicks denied access to feed area N3: chicks denied access to perches and dark area	22	N1 more optimistic than N2	Seehuus et al. (2013)[273]
	Go/No-Go	Spatial & visual	Approach	JB Test #1: N1: simple environment N2: simple environment + cold stress P1: complex environment P2: complex environment + cold stress JB Test #2: All treatments exposed to unpredictable added stressors	87	No effects during JB Test #1 P1 & P2 remained optimistic during JB Test #2, while N1 & N2 were less optimistic during JB Test #2	Zidar et al. (2018)[275]

Species	Test type	Cue	Response	Affect manipulation	N tested	Result	Reference
	Go/No-Go	Visual	Approach	N1: 5-min social isolation N2: 60-min social isolation C: no social isolation	40	N1 more pessimistic, N2 more pessimistic and less optimistic	Salmeto et al. (2011)[237]
Fish	Go/No-Go	Spatial & visual	Approach	N: housed with non-preferred male P: housed with preferred male	35	N more pessimistic	Laubu et al. (2019)[276]
	Go/No-Go	Visual	Approach	N: barren tank with strong light P: enriched tank with gentle light	96	P more optimistic	Wojtas et al. (2015)[277]
	Go/No-Go	Spatial	Approach	N: housed with a larger, aggressive conspecific C: housed in isolation	6	N more pessimistic	Rogers et al. (2020)[278]
Broiler chickens	Go/No-Go	Spatial	Approach	N: corticosterone-treated birds C: control	14	N more pessimistic	Iyasere et al. (2017)[290]

Active choice (+) = active choice task with positive reinforcement

N = perceived negative affect-inducing treatment

C = control treatment

P = perceived positive affect-inducing treatment

The first study utilizing a judgement bias test in animals was conducted by Harding et al. (2004)[270]. Rats were trained on a Go/No-Go task to discriminate between two tones, each associated with a lever – one tone, if played and the lever was pressed, resulted in a reward and the other tone, if played and the opposing lever was pressed, resulted in

nothing. Afterwards, rats were housed under either normal or poor housing conditions for 9 days. During testing, they played ambiguous tones (tones intermediate to those previously played during training). Rats housed under poor conditions were approximately 1s slower to respond to the ambiguous tone closest to the positive tone and approximately 2s slower to respond to the positive tone than rats housed under normal conditions. This indicates that rats housed in normal conditions were more optimistic that they might receive a reward in ambiguous situations than rats housed in poor conditions.

There are documented limitations to judgement bias tests which include: time-consuming training phases[224], selection bias[222], and acquisition effects[257]. The time required to train subjects to discriminate between cues can be lengthy, resulting in impractical application of the test in commercial settings and loss of subjects eligible to be tested. Typically, judgement bias tests are administered after affect manipulation. Stressful manipulations of affective state have been shown to reduce learning ability, therefore creates a selection bias against animals in negative affective states that did not meet the training criterion[215,224,291]. Theoretically, the subjects to be tested might not be a representative sample of the entire population, as subjects in negative versus positive affective states are excluded or included disproportionately due to differences in learning ability. Lastly, subjects that are tested repeatedly can learn that ambiguous cues are not reinforced, therefore reduce their likelihood of responding[215,224]. This issue has been demonstrated in literature, where sheep tested repeatedly showed a decline in total number of approaches to ambiguous cues over 3 weeks, suggesting they learned these cues were unrewarded[257].

2.5.2 Attention bias tests

Attention biases have been described as "the differential allocation of attentional resources towards one stimulus compared to others"[224]. In humans, anxiety is considered an affective state disorder, and results in an increased focus on negative information[224]. For instance, anxious individuals are more likely to bias their attention

towards threatening stimuli than non-anxious individuals[221,292]. This increased anxiety can be quantified by observing vigilance behavior (e.g., visual scanning, alertness), where vigilance is more frequent in threatening situations and infrequent in non-threatening situations. Indeed, when in the presence of a threatening situation (aversive handling), cattle spend more time with their head upright than when exposed to a neutral situation (gentle handling)[293]. A review of affect-driven attention bias studies discovered that of the 12 identified studies, 11 found effects of treatment on attention bias in animals[224]. Therefore, attention biases can be used as an indicator of how animals perceive their environment, and therefore, their welfare[224].

The most common form of attention bias testing is called the "attentional probe" or "dot probe" task[294]. In this method, two stimuli are presented simultaneously, one positive (e.g. feed) and one negative (e.g. a conspecific call indicating a potential threat)[294]. Thereafter, observation of the subject's behavior can indicate whether their attention is biased towards the positive or negative stimulus. For example, a longer latency to begin feeding coupled with increased vigilance behavior could indicate an animal whose attention is biased towards the negative stimulus (potential threat) rather than towards the positive stimulus (feed), and therefore in a negative affective state. On the other hand, a shorter latency to begin feeding and decreased vigilance behavior would indicate an animal whose attention is biased towards the positive stimulus (feed) compared to the negative stimulus (potential threat), and therefore in a positive affective state. Because training is not required, or in some cases is much less time consuming, attention bias tests can be more practically applied to assess affective state compared to judgement bias tests[224]. Studies evaluating affective states of livestock species are summarized in Table 2.4.

Table 2.4. Summary of studies evaluating affective state of livestock species through an attention bias test.

Species	Stimuli	Affect manipulation	Measure of attention	N	Result	Reference
Pigs	Feed bucket/ flashing light + moving door	1: enriched 2: barren 3: enriched to barren 4: barren to enriched	Attention towards threat, vigilant behavior, latency to feed, time spent feeding, vocalizations, defecations and urinations	128	No effect on attention towards threat, vigilant behavior, or time spent feeding; Latency to feed 1 & 4 < 2 & 3; Vocalizations 1 & 4 > 2 & 3; No effect on defecation or urination	Luo et al. (2019)[295]
Cattle	Hay/dog	N: anxiogenic drug P: anxiolytic drug C: saline	Attention towards threat, vigilant behavior, zones crossed, vocalizations, latency to feed, time spent feeding, urinations and defecations, ear posture, head shaking, tail swishing	36	Attention towards threat/vigilant behavior/backwards ear posture N > C & P; Latency to feed N > C & P; Time spent feeding C & P > N; Head shaking/tail swishing/defecating/zones crossed N > C & P	Lee et al. (2018)[296]
Laying hens		N: anxiogenic drug C: saline	Latency to first step/vocalize/feed following first and second alarm call, number of steps and vocalizations, time spent feeding	50	Latency to eat following first and second alarm call N > C; No effect on latency to first step/vocalize following first alarm call or latency to first step following second alarm call; Latency to first vocalize following second alarm call C > N; Number of steps/vocalizations N > C; Time spent feeding C > N	Campbell et al. (2019)[297]
	Mixed grain/ conspecific alarm call	N: indoor ranging hens P: outdoor ranging hens	Latency to step, vocalize, and feed following an alarm call playback (hens required to feed before the alarm call was played)	67	Latency to step N > P; Latency to vocalize P > N; Greater number of N hens did not eat at all (never received an alarm call playback) compared to P hens; Of those N hens that did initially feed, only 7% resumed feeding after the alarm call was played compared to 36% of P hens	Campbell et al. (2019)[298]

Species	Stimuli	Affect manipulation	Measure of attention	N	Result	Reference
Sheep	Photograph of a sheep/dog	N1: anxiety-inducing drug N2: depression-inducing drug C: saline	Attention towards both stimuli, vigilant behavior, number of vocalizations and zones entered, number of times and latency to sniff the photograph or floor/walls of arena	48	N2 most attention towards threat & least attention towards photo; N1 most attention towards photo & least attention towards threat; Vigilant behavior/number of times and latency to sniff photo N1 & N2 > C; Number of times and latency to sniff arena/zones entered N2 & C > N1; Vocalizations C > N1 & N2	Monk et al. (2018)[299]
	Feed bowl/ dog	N: rest disruption and individual housing C: group housing	Vigilant behavior, latency to feed, amount of feed eaten	60	Vigilant behavior C > N; Latency to feed C > N; Amount of feed eaten N > C	Verbeek et al. (2019)[287]
	Feed bucket normally used for feeding	N: low-feeding P: high-feeding	Latency to first detection and approach stimulus, time spent interacting with stimulus, vocalizations	41	No effect on latency to first detection and approach stimulus or vocalizations; Time spent interacting with stimulus N > P	Verbeek et al. (2014)[261]
	Photograph of a sheep/dog	N: anxiety-inducing drug P1: calm-inducing drug P2: happy-inducing drug C: saline	Attention towards both stimuli, vigilant behavior, number of vocalizations, number of sniffs of photo and environment, zones crossed, urinations	80	Vigilant behavior N > C, P1, & P2; No effect on attention towards dog or vocalizations; Number of sniffs of photo and environment N < C, P1, & P2; Zones crossed P1 & P2 > N; Urinations N > C, P1, & P2	Monk et al. (2020)[300]
	Feed bucket/ dog	N: anxiogenic drug P: anxiolytic drug C: saline	Attention towards threat, vigilant behavior, zones crossed, number of vocalizations, latency to feed, time spent feeding	60	Attention towards threat/vigilant behavior N > C > P; no effect on zones crossed or vocalizations; no N sheep fed, with no difference between P & C	Lee et al. (2016)[301]
	Hay/dog	N: anxiogenic drug P: anxiolytic drug	Attention towards threat, vigilant behavior, zones crossed,	60	Attention towards threat N & C > P; vigilant behavior N > C & P; no effect on zones crossed or vocalizations; No N sheep fed,	Monk et al. (2018)[302]

Species	Stimuli	Affect manipulation	Measure of attention	N	Result	Reference
		C: saline	vocalizations, latency to feed, time spent feeding		no difference between P & C	
	Feed bowl/dog	N: chronic stress C: group housing	Attention towards threat, vigilant behavior, latency to feed, zones crossed, freezing behavior	32	No effect	Monk et al. (2019)[303]
	Sheep bleating/dog barking (recordings)	N: negative mood (i.e. various unpredictable/ uncontrollable events) P: positive mood (i.e. conditions better than minimal commercial standards)	Head and ear positions and movements	31	N sheep shifted their attention towards dog barking when it was at a lower volume than the sheep bleating; N sheep shifted their attention towards the sheep bleating when both recordings were of the same volume; P sheep shifted attention towards dog barking when both recordings were of low intensity	Raoult & Gygax (2019)[304]

Chapter 3. Environmental complexity, but not stocking density, positively impacts affective states of broiler chickens

Anderson, M.G.[1], Campbell, A.M.[1], Crump, A.[2], Arnott, G.[3], Jacobs, L.[1*]

[1]Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Virginia, USA

[2]Centre for Philosophy of Natural and Social Science, London School of Economics and Political Science, London, UK

[3]School of Biological Sciences, Queen's University Belfast, Belfast, UK

*Corresponding author: jacobsl@vt.edu

3.1 Abstract

Affective state can bias an animal's judgement. Animals in positive affective states can interpret ambiguous cues more positively ("optimistically") than animals in negative affective states. Thus, judgement bias tests can determine an animal's affective state through their responses to ambiguous cues. We tested the effects of environmental complexity and stocking density on affective states of broiler chickens through a multimodal judgement bias test. Broilers were trained to approach reinforced locations signaled by one color and not to approach unreinforced locations signaled by a different color. Trained birds were tested for latencies to approach three ambiguous cues of intermediate color and location. Broilers discriminated between cues, with shorter latencies to approach ambiguous cues closest to the reinforced cue than cues closest to the unreinforced cue, validating the use of the test in this context. Broilers housed in high-complexity pens approached ambiguous cues faster than birds in low-complexity pens–an optimistic judgement bias, suggesting the former were in a more positive affective state. Broilers from high-density pens tended to approach all cues faster than birds from low-density pens, possibly because resource competition in their home pen

increased food motivation. Overall, our study suggests that environmental complexity improves broilers' affective states, implying animal welfare benefits of environmental enrichment.

Keywords: environmental enrichment, stocking density, affective state, optimism, judgement bias, animal welfare

3.2 Introduction

Broiler chickens are typically housed in barren environments and at high stocking densities in order to minimize production cost, which has the potential to compromise broiler welfare[127,305–307]. In conventional housing systems, broilers spend approximately 80% of their time budget lying down, and a positive association between time spent lying down and lameness has been found, which negatively influences broiler welfare[40]. Environmental enrichment (complexity) has a positive impact on animals' biological functioning and behavior[127,157,164,165,275,307,308]. Broilers with access to elevated platforms experienced improved gait through an increased occurrence of low (good) gait scores, lower (better) flock mean gait score, and lower occurrence of tibial dyschondroplasia compared to broilers without access to platforms[150]. Stocking density is another important environmental factor that can impact broiler chicken welfare aspects, such as leg health, level of bruising and scratches, lameness, and behavioral suppression[31,47,59]. While housing broilers at high stocking densities maximizes profit for the producer, it has the potential to compromise bird health and welfare as seen through decreased final body weight and feed conversion ratio, as well as increased occurrence of footpad dermatitis and mortality[24,47,230,309]. Furthermore, high stocking densities reduce space use and therefore activity level, and increase disturbances that lead to decreased plumage and carcass quality[49,50,305,309,310]. These and other studies show that conventional housing of broiler chickens has some negative effects on bird health and welfare, although their effects on affective state are unknown.

The ability to perform highly motivated behaviors is important for good animal welfare[60,127,169,311]. Three species-specific behaviors have been identified for broilers, whose deprivation may cause negative affective states: perching, dustbathing, and foraging[60–64]. Perching is a natural behavior for jungle fowl (broilers' ancestors), which seek elevated resting spaces possibly to avoid predation while sleeping[56,65]. Broilers have maintained motivation to perch when given easily accessible perches[67]. When provided perches at a low stocking density, broilers perched for longer (nearly 25% of the observation periods) and were less aggressive than control birds[157]. Dustbathing is another highly-motivated behavior for broilers, as jungle fowl who are deprived of substrate to dustbathe in will exhibit excessive, compensatory dustbathing behavior when eventually provided with a substrate or even dustbathe on wire flooring, highlighting the need to perform this behavior regardless of access to a suitable substrate[70]. Broiler chickens dustbathe throughout their life, even chicks have been shown to dustbathe during the first week of life[312] (Jacobs, 1.7% of observed time, unpublished data). Furthermore, jungle fowl and broilers have retained the motivation to forage. Even when regularly fed, semi-wild junglefowl foraged for 61% of the time they spent active, as well as spent 34% of their time scratching, a behavior considered to be associated with foraging[41]. Without access to a preferred substrate or environment, domestic fowl forage in feces, suggesting that this behavior is highly motivated[74,75]. Commercial broilers can be deprived of all three of these high-motivation behaviors; they do not have access to perches or proper substrate to dustbathe and forage in. While they do have access to litter, this is often reused between flocks, and therefore contains moisture and feces that fosters rapid bacteria growth and ammonia production[75,77]. Because broilers are inactive for large portions of the day, the risk of developing contact or footpad dermatitis increases with provision of dirty litter, and could lead to a negative affective state due to pain associated with dermatitis[79,80,313].

Life experiences, including environmental conditions, can elicit short-term emotions which are defined as functional states elicited by reward or punishment (stimuli that animals work to either gain or avoid)[14]. These emotions are adaptive and help animals appropriately respond to changes in their environment[211]. Emotional responses shape an animal's mood, which can be defined as long-term, diffuse states that reflect the

cumulative valence of emotions over time[13]. Both emotion and mood contribute to an animal's affective state, which can be measured along a spectrum between positive and negative (valence)[13]. An animal that experiences more positive than negative emotions throughout life, for instance induced by the ability to express play behaviors, will be in an overall positive affective state, compared to animals that have more negative experiences[211]. When an animal experiences more negative than positive emotions, such as chronic or excessive fear and anxiety, the animal will be in an overall negative affective state[165,314,315]. Typically, negative experiences tend to have a stronger influence on affective state than positive experiences[230,316,317]. In order to achieve good welfare, the induction of positive experience must be considered in addition to preventing negative experiences. However, most published studies for agricultural species focus on avoidance of negative affective states such as level of fear[155,164], or physiological measures, like lameness[153,308,318].

Affective states are closely associated with cognition–the mechanisms by which animals acquire, process, store, and act on information from their environment[211]. Affective states influence cognitive processing and cognitive processing impacts affective states[212–214]. When emotions and affective states impact aspects of cognition, such as judgement, attention, and memory, we call this "cognitive bias"[215,223,224]. Cognitive biases can be used as an indicator of animal welfare[211,215,227,228].

Humans experiencing a negative affective state (depression, anxiety) tend to interpret ambiguous events negatively and have a pessimistic outlook, whereas humans who are in a more positive affective state tend to interpret the same ambiguous events positively and have a more optimistic outlook[319–322]. Animal responses to ambiguous situations can be quantified using a judgement bias test, assessing cognitive bias. Judgement bias testing is used to determine levels of optimism and pessimism of subjects based on responses to ambiguous cues during testing. Judgement bias cues can be spatial[236], visual[237], auditory[238], olfactory[239], tactile[240] or a combination of these (multimodal[227]). Shorter latencies to approach ambiguous cues would indicate optimism, whereas longer latencies to approach ambiguous cues would indicate pessimism[211,215,219,241,247,270,275,292]. A meta-analysis of 71 judgement bias studies on 22 species showed optimistic and

pessimistic responses to ambiguous situations resulting from positive or negative affective states, respectively[235]. Thus, judgement bias is considered the "gold standard" for evaluating affective states in animals[323].

One previous study has evaluated broiler chicken affective state through a judgement bias test. Iyasere et al. (2017)[290] trained broilers on a spatial, go/no-go judgement bias task to discriminate between a reward- (mealworms) and punishment-associated (air puff) cone. Following discrimination, birds treated with corticosterone had longer latencies to displace cones at all cue locations compared to control birds, suggesting a pessimistic bias. A similar test approach was conducted on laying hens, where 'exploratory' layer hens (categorized based on novel object and open area test responses) housed with enrichments showed more optimistic responses than exploratory hens housed without enrichments[324]. Therefore, judgement bias tests could be a valuable tool to assess positive affective states in broilers housed under varying environmental conditions. Including an evaluation of lameness in conjunction with judgement bias testing is warranted, as birds were required to walk in judgement bias training and testing.

In the present study, we used a judgement bias test to assess the effect of environmental complexity and stocking density, manipulated in a factorial experiment, on broilers' affective states. Gait was quantified as a potential confounding factor for the judgement bias test. We hypothesized that birds housed in high-complexity (HC), low-density (LD) environments would respond more optimistically, indicating more positive affective states, compared to birds from low-complexity (LC), high-density (HD) environments. Birds housed in LC/LD or HC/HD environments were predicted to show intermediate levels of optimism.

3.3 Results

3.3.1 Judgement bias test

Training for the judgement bias task took between 3-10 sessions (median 4 sessions), with birds learning the task after 30 days of training. Out of 36 birds that began training, 9 passed the learning criterion to move on to testing (3 from low-complexity/high-density [LC/HD], 3 from high-complexity/low-density [HC/LD], 2 from high-complexity/high-density [HC/HD], and 1 from low-complexity/low-density [LC/LD] pens). Birds from LC/HD, HC/LD, HC/HD, and LC/LD pens learned the task after a mean of 6, 7, 8, and 10 training sessions, respectively.

Testing round tended to impact latencies to approach all cue types ($F_{1,3} = 2.35$; $p = 0.074$). Latency to approach (s) all cues was 32.96 ± 3.14s in test round 1, 40.43 ± 3.16s in test round 2, 36.62 ± 3.14s in test round 3, and 39.69 ± 3.16s in test round 4. There was no effect of reward side (left or right) or color (black or white) on latencies to approach during the judgement bias test ($F_{1,7} = 3.42$; $p = 0.107$).

Across environmental complexity and stocking density treatment groups, birds approached the P (15.08 ± 2.08s) and NP (20.13 ± 3.07s) cues faster than the MID (44.19 ± 3.07s), NN (54.27 ± 3.07s), and N cue types (58.67 ± 2.57s; $F_{2,239} = 73.64$; $p < 0.001$; Figure 3.1). Birds from high-complexity pens had a shorter mean latency to approach all cues than birds from low-complexity pens ($F_{1,6} = 16.816$, $p = 0.006$). Additionally, birds from high-complexity pens approached the NN cue faster ($F_{1,35} = 4.934$, $p = 0.033$; Figure 3.2) and tended to approach the P cue faster ($F_{1,89} = 4.03$; $p = 0.085$) than birds from low-complexity pens, but no differences between enrichment treatments were found for the P NP ($F_{1,35} = 1.508$, $p = 0.268$), MID ($F_{1,35} = 1.325$, $p = 0.261$), or N cues ($F_{1,53} = 1.35$; $p = 0.283$). Birds from high-density pens showed a trend to approach cue types faster than birds from low-density pens ($F_{1,6} = 5.767$, $p = 0.053$; Table 3.1). Pairwise differences in latency to approach cues were not found for the density treatment. No interaction effect of enrichment and stocking density was found on latency to approach cue types.

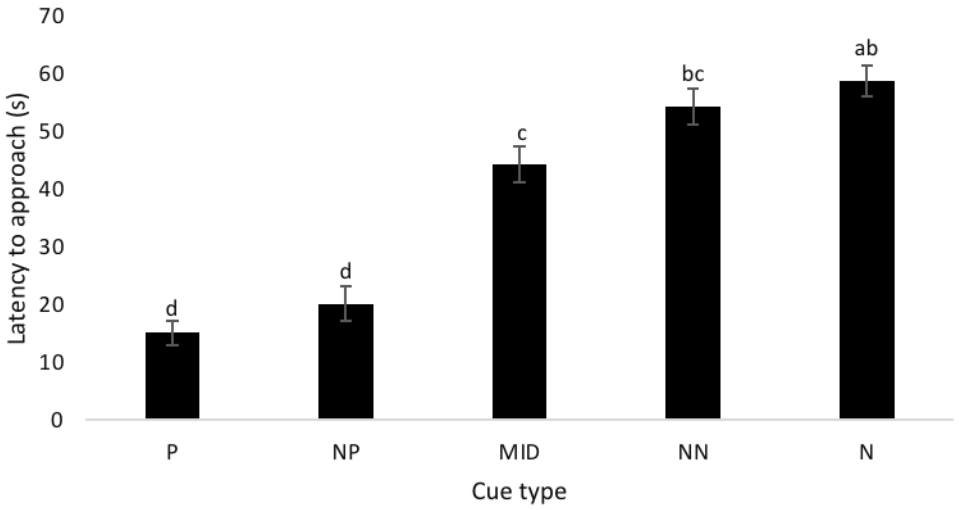

Figure 3.1. Least squares mean estimates (±SEM) for latency to approach (s) all five cues (positive [P], near positive [NP], middle [MID], near neutral [NN], and neutral [N]) in the judgement bias test for 4 test rounds (n = 9). Means with different superscripts ($^{a-d}$) differ at $p < 0.001$.

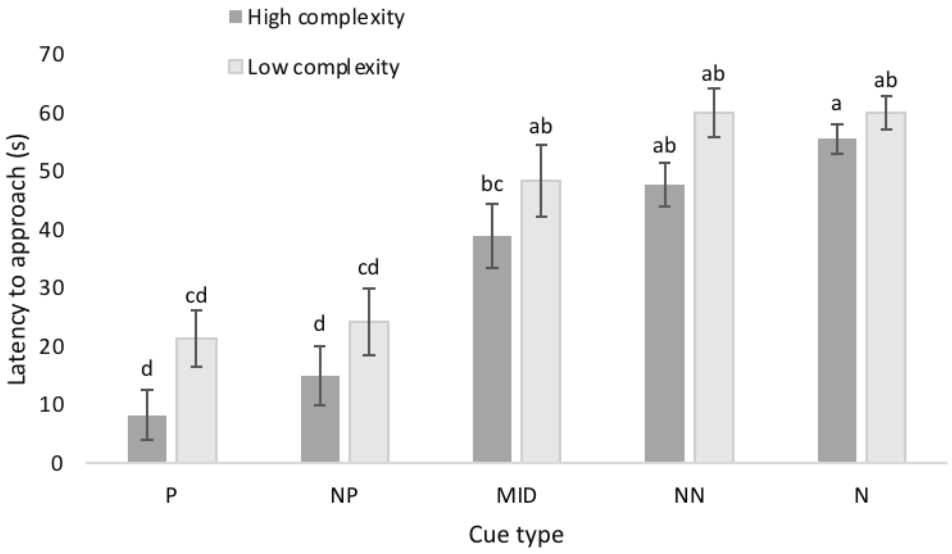

Figure 3.2. Mean (± SEM) latency to approach (s) all five cues (positive, P; near positive NP; middle, MID; near neutral, NN; and neutral, N) for birds from both high- and low-complexity pens in the judgement bias test for 4 rounds (n = 9). Means with different superscripts ($^{a-d}$) differ at $p < 0.01$.

Table 3.1. Mean (±SEM) latency to approach (s) all cues (positive, P; near positive, NP; middle, MID; near neutral, NN; and neutral, N) of birds from both high- and low-density pens in the judgement bias test for 4 rounds (n = 9).

	Stocking Density	
	High[1]	Low[2]
Cue type	Mean latency ± SEM (s)	Mean latency ± SEM (s)
P	11.21±5.22	17.41±5.83
NP	17.57±5.61	20.83±6.27
MID	40.03±5.39	46.89±6.02
NN	53.04±4.04	53.31±4.52
N	59.05±2.62	55.69±2.93

[1]42.08kg/m^2 at day 50

[2]23.83kg/m^2 at day 50

3.3.2 Gait

No effects of enrichment or stocking density treatments were found on bird gait score ($p > 0.1$). Mean gait scores were 0.125 ± 0.045 for high-density, 0.014 ± 0.014 for low-density, 0.014 ± 0.014 for high-complexity, and 0.125 ± 0.048 for low-complexity pens. Age tended to affect gait scores, with 93.1% of birds receiving a score 0, 6.9% a score 1, and 0% a score 2 on day 19, compared to 95.8% receiving a score 0, 1.39% a score 1, and 2.78% a score 2 on day 33 ($F_{1,143} = 2.983$, $p = 0.094$; Figure 3.3). Gait score was not associated with latency to approach during the judgement bias test ($\chi^2 = 0.982$, $p = 0.621$).

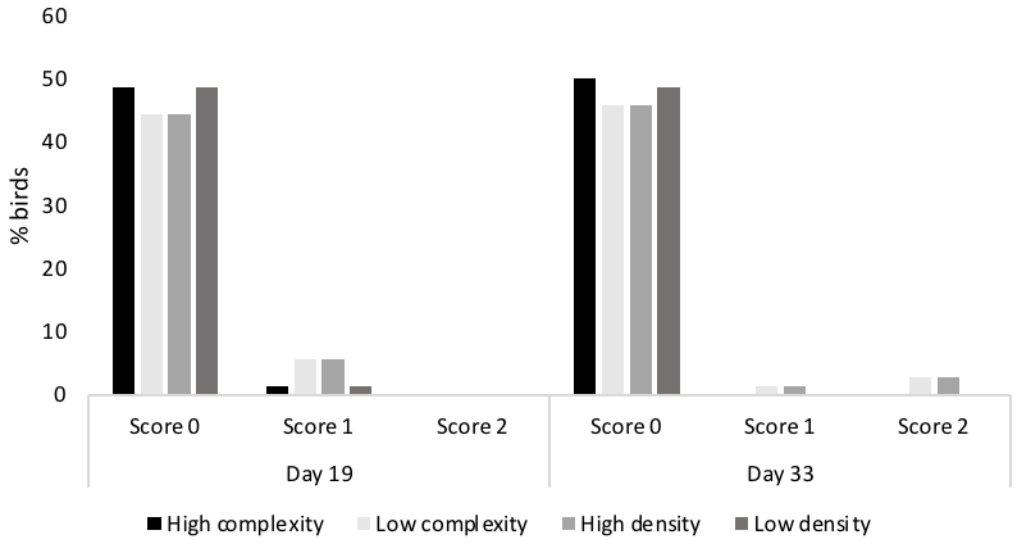

Figure 3.3. Percentage of birds (observation n = 144) in high-complexity, low-complexity, high stocking density (42.08kg/m^2), and low stocking density (23.83kg/m^2) pens receiving a gait score of either 0, 1, or 2.

3.4 Discussion

This study is the first to apply a judgement bias test to evaluate affective state of broiler chickens housed in varying environmental conditions. Affective state was manipulated by placing birds in either high- or low-complexity pens under either a high or low stocking density. Birds were trained to discriminate between multimodal cues, with one color and location associated with a mealworm reward and the opposite color and location unreinforced. Within a relatively limited time frame (approximately 5 weeks), 25% of the selected birds successfully learned to discriminate between the P and N cues. All birds approached the P and NP cues quicker than the MID, NN, and N cues. Birds from HC pens were faster to approach all cue types than birds from LC pens and had a shorter latency to approach the NN cue than birds from LC. Additionally, birds from HD pens tended to approach all cue types faster than birds from LD pens.

In this study, we validated a judgement bias test as an appropriate welfare indicator for broilers. During testing, birds showed longer latencies to approach cues closer to the N

cue and shorter latencies for cues closer to the P cue (a generalization gradient[211,241]). The generalization gradient of bird responses shows that broiler chickens can learn a discrimination task and that the judgement bias test is a valid tool to assess affective states in broiler chickens. One methodological issue with judgement bias tests is that subjects can learn that ambiguous cues are unreinforced, causing a reduction in responsiveness that gives the appearance of increasing pessimism despite no change in affective state[211,257]. In line with this, test round showed a trend to impact latency to approach cue types, although latencies did not show a linear increase over time.

Enrichment that allows broilers to express highly-motivated, natural behaviors improves their welfare[127,211]. Lower stocking densities compared to commercial standards enables broilers to perform more species-specific behaviors associated with environmental enrichment[325]. In line with our hypothesis, birds from high-complexity pens showed shorter latencies to approach all cues compared to birds from low-complexity pens. This faster approach suggests birds from the complex environment had a greater expectancy to receive a reward compared to birds from the barren environment. Additionally, birds from complex pens approached the NN cue faster than birds from barren pens (12s difference). This interaction between enrichment and cue type suggests that birds from complex pens were more optimistic than birds from barren pens. However, approaches did not differ for the two other ambiguous cues (NP and MID), suggesting some association between enrichment and affective state (optimism). Our findings are similar to laying hen responses[324], where 'exploratory' layer hens housed with preferred enrichments flipped an ambiguous cue lid more often than exploratory hens housed without enrichments (81% vs. 55% of ambiguous lids, respectively). Although the task was different for broilers in our study compared to their layer hens, both studies suggest that environmental enrichment positively impacts affective states (optimism).

Birds from high-density pens tended to be faster to approach all cues compared to birds from low-density pens. This result was opposite to our predictions, as previous broiler studies have found that high stocking densities lead to fear[32] and stress[326], reduced ability to perform species-specific behaviors such as perching[157], and a higher rate of

aggression and disturbances of resting birds[157,172]. Our results may be attributed to birds housed at high densities having to compete for resources within their home pens, resulting in an increased motivation to receive a food reward during judgement bias training and testing. In support of this explanation, broilers housed at high rather than low stocking densities showed decreased body weights[327], growth performance[326], and feed intake[328], possibly due to limited access to feeders[329]. Future research might overcome this limitation by implementing a judgement bias test that does not use feed as a reinforcer, but instead visual cues[237].

One limitation of our study is that only 9 out of 36 subjects that began training learned the judgement bias task. This may be because our paradigm required birds to walk towards a cue. Broiler chickens are prone to impaired gait, difficulty walking, and lameness as they reach slaughter weight and age[30,52,80,330]. This could have affected our subjects' ability to meet the training criterion, resulting in a low sample size for testing. This explanation is supported by our results showing that gait score increased (worsened) across the study, indicating a worse gait on day 33 than day 19 (Figure 3.3). On either day, however, most birds had low gait scores (95.8% receiving score 0 on day 33), suggesting that gait did not impair birds' ability to approach cues during judgement bias testing. It is nonetheless possible that even birds with low gait scores could have struggled to walk due to body weight or size. This is supported by results from Bokkers & Koene[331,332], where fast-growing broilers with low gait scores (good gait) were slower to reach the end of a runway compared to slow-growing broilers at 12 weeks of age (150s versus 70s). Training methods need to be refined in order to increase the total number of birds successfully passing the learning criterion. Altering the testing methodology to a pecking task could be a possible solution to these limitations, as evaluated in laying hens[241]. Broilers could be trained to peck at a cue within close proximity, therefore removing the need for locomotion, but still retaining the discrimination response (latency to peck).

This study validated judgement bias tests as a novel approach to assess affective state in broiler chickens, with environmental enrichment increasing optimism consistent with a beneficial effect of enrichment on broiler affective state. Although 75% of birds did not

learn the task within 30 days, we demonstrated optimistic responses of the 9 learned birds due to the high-complexity treatment. Thus, judgement bias tests are a promising indicator of affective state in broilers.

3.5 Materials and Methods

Ethics

This experiment was approved by Virginia Tech's Institutional Animal Care and Use Committee (approval number: 19-175), and animal welfare as prioritized throughout. All methods were carried out in accordance with relevant guidelines and regulations.

Animals and housing

We conducted this experiment at Virginia Tech's poultry facility during February and March 2020. Male Ross 708 chicks (n = 1620) were obtained at day 0 from a commercial hatchery (PA, USA) where they were vaccinated for Marek's disease, followed by transportation to the research facility. Upon arrival, chicks were randomly allocated to one of four treatment groups in a 2×2 factorial design using environmental complexity and stocking density as factors at pen level. Each treatment group was replicated three times (12 pens total) randomly distributed in a block design. Pens (14.5m^2) contained standard pine shavings as bedding (approximately 10.15cm depth), four feeders, and three water lines with nipple drinkers. All birds had ad libitum access to water and commercial broiler chicken feed (starter day 0-14, grower day 15-28, and finisher day 29-50). Birds received heat lamps and 24h light in the first 7 days, followed by a light:dark schedule of 18L:6D thereafter, with a light intensity of approximately 15 lux during light hours. Due to a technical issue, birds received 24h light for 7 additional days during week 2 of age. House temperature was gradually decreased from 35°C on day 1 to 21°C on day 50 by assessing bird comfort. All birds received a therapeutic dose of antibiotics via the water lines from day 33-40 in response to a disease outbreak that resulted in an increased cull and mortality rate due to pathogen exposure.

Environmental complexity

Six pens provided a high-complexity (HC) environment, while the other six pens provided a low-complexity environment (LC), similar to commercial standards. HC pens contained four functional spaces, including space for 'feeding' (approximately $3m^2$), 'comfort' (approximately $3m^2$), 'resting' (approximately $3m^2$), and 'exploration' (approximately $4.3m^2$). The 'feeding', 'comfort', and 'resting' spaces included a water line with three nipple drinkers. The feeding space contained four feeders and one third of a medium PECKstone™ (Proteka Inc., VILOFLOSS, Germany) broken into smaller pieces. The comfort space contained a wooden-frame dust bath (180cm x 91cm x 10cm) filled with 68kg of playground sand (QUIKRETE, GA, USA). Sand was raked and partially replaced when deemed necessary. The resting space included three perches (182.9cm L x 30.5cm W x 8.5cm H) modified from LeVan et al. (2000)[333] and Pettit-Riley and Estevez (2001)[172], using 1.91cm-diameter PVC pipe, which was sprayed with textured black spray paint (Rust-Oleum, IL, USA) to enhance grip while perching. Birds had access to 7.6cm of horizontal perch space/bird in high stocking density pens and 15.2cm in low stocking density pens. The exploration space contained a pair of enrichments. Six enrichments ("toys") were randomly paired into three groups of two, combining a nutritional and occupational enrichment starting on day 2. These enrichments were rotated every three days according to a randomized schedule to maintain variation and novelty.

The LC pens provided the same structural space as HC pens, but without enrichments. Four feeders and three drinker lines were distributed throughout the pens.

Stocking density

Six pens (LC n = 3; HC n = 3) were stocked at a high-density (HD) of $42.08kg/m^2$ at day 50, with 180 chicks/pen on day 0. The other six pens (LC n = 3, HC n = 3) were stocked at a low-density (LD) of $23.83kg/m^2$ at day 50, with 90 chicks/pen on day 0.

Judgement bias test

On day 2, 36 chicks (n = 3/pen) were wing banded and gently marked on the upper back with black livestock marker (All-Weather Paintstik, LA-CO Industries, Inc., IL, USA). These markings were reapplied as necessary throughout the experiment. The judgement bias test followed a 4-step process, including habituation, phase 1 training, phase 2 training, and testing. All steps of the judgement bias test were video recorded (EOS Rebel T7 DSLR Camera, Canon). The test was performed in an arena (plywood 122cm x 61cm with rubber interlocking mats as flooring) and conducted between 07:00-13:00h. On day 10, the arena was shortened to 91cm to ensure ease of walking to the far end as the birds aged.

Birds were first habituated to the judgement bias arena by a single observer from day 2-11. The arena contained four arbitrarily-placed cardboard feed flats (5 x 5cm) filled with a reward (dried mealworms) and two empty black and white containers (4oz; Ziploc®, S.C. Johnson & Son, Inc.). During habituation, 3 birds from the same pen were placed in the arena for 6 rounds (1 round/day), with each round lasting 5 minutes. For the first round of habituation, birds were placed directly into the arena with the start box door closed, so birds could not access the start box. For the second habituation round, birds were placed into the start box with the door open so that the arena was clearly visible and accessible. During the following four rounds, birds were placed into the start box with the door closed so birds could not see the arena, and then the door was opened immediately after the third chick was placed into the start box so that the arena was clearly visible and accessible. During round 6, feed flats with mealworms were moved to the far end of the arena from the start box and latency to eat was recorded. Birds were considered habituated when they consumed a mealworm during any of the rounds. All chicks consumed a mealworm at least once after round 6.

Phase 1 of training began on day 13 and was performed by two experimenters. Birds were individually conditioned to walk to the far end of the arena from the start box using a rewarded cue. Birds were individually trained to associate a color cue (100% black or white; n = 17, n = 19) and location (right or left, n = 17, n = 19) with a reward (dried mealworms; both cues counterbalanced). Rewarded cues and locations were balanced across treatments. The color cues (12.2cm W x 25.4cm L photo paper) were

taped on the far wall of the arena at the preassigned rewarded location (left or right). The arena was divided into 15cm sections numbered 1 (closest to start box) through 6 (furthest from start box) to record distance walked.

Each phase 1 training session lasted 6min, with the bird allotted 1min to approach the rewarded container. A plastic black or white container with mealworms was initially placed at section 1 (approx. 15cm from start box opening) and moved back to section 2 (approx. 30cm from start box opening) on the following attempt if the bird approached the container at section 1. This continued until the bird successfully approached the container at section 6 within 1min. After each 1min attempt, the observer gently picked up the bird 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. Birds either would approach the container and eat, after which the container was moved back to a further section, or they would not, after which the attempt was repeated with the container placed at the same section. If the bird did not approach the container after three attempts, the container was moved one section closer to the start box. The observer recorded frequency and latency to approach the container (time from opening start box door until the bird's head was over the container) and whether the bird ate mealworms (yes/no) from the container. Birds continued to phase 2 of training when they ate mealworms from the container at either all 6 sections consecutively, or at section 6 for all attempts within one session (6 attempts). The first bird reached the learning criterium in session 3 and the last bird in session 10.

Phase 2 of training began on day 23; all training was performed by two experimenters. Positive and neutral cues were presented individually at section 6 according to a pseudorandomized order with no more than two of either cue presented consecutively. Experimenters live-recorded latency (s) to approach cues and whether the bird ate mealworms (yes/no) from the container. Birds continued to the testing stage when they approached the rewarded cue on their own within 1min 100% of the time that it was presented in a single session and did not approach the neutral cue within a single session consisting of 6 attempts. Inter-observer reliability for all measures of pre-training and training showed a high level of agreement (simple Kappa Coefficient between 0.91

and 1 for approach (1/0), latency to approach (s), eating mealworms (1/0), and distance walked (0-6).

Depending on when birds met the phase 2 learning criterion, testing occurred from day 39 to day 50 and was performed by a single experimenter, during which the positive (P), neutral (N), and three ambiguous cues (near positive, NP; middle, M; near neutral, N) were individually presented at intermediate locations (75% black/near right, 50% black/middle, and 25% black/near left). Each bird that advanced to testing (n = 9) was tested four times over two days, with 45-60min in between testing sessions on a single day. Testing sessions lasted a maximum of 7min, with 1min attempts for the bird to approach each presented cue (total n = 28 attempts/bird). Cues were presented in a pseudorandomized order. The first and last attempt in a testing session were always rewarded to maintain motivation throughout the test. All other cues were unreinforced. The experimenter live-recorded frequency and latency to approach cues. A ceiling latency score of 60s was scored for trials when the bird did not approach the cue.

Gait

Individual broiler gait score was assessed by a single observer on day 19 and day 33 on 3 birds/pen (n = 36), with the same 36 birds scored on both days. On each gait scoring day, every bird was scored twice (4 observations/bird). The observer entered the birds' home pen and gently encouraged an individual bird to walk 1.5m. A plastic PVC pipe was used to herd the subject to a clear path in the home pen and increase distance between the observer and subject. The subject was then given a score out of three categorical descriptors (0-2) from Webster et al. (2008)[334]. Score "0" indicated the subject was able to walk at least 1.5m with no obvious impairment and a balanced gait; score "1" was given to subjects able to walk at least 1.5m, but showing obvious impairment with a clear limp or awkward gait; and score "2" indicated that the subject was unable to walk 1.5m, showing severe impairment with or without shuffling on the shanks or hocks with assistance of wings.

Statistical analysis

Data were analyzed in JMP pro 15 (SAS Institute Inc., Cary, NC, USA). Residuals were deemed normally distributed based on visual inspection of normal quantile plots. For the judgement bias data, we ran general linear mixed effect models with latency as the response variable; enrichment, stocking density, and cue position as fixed effects; and bird ID as a random effect. Interactions between the fixed effects were included in the model and removed if not significant. A Tukey Kramer's pairwise comparison post-hoc test was used to compare latencies to approach each cue type, with enrichment*cue as fixed effects and bird ID as a random effect. To check for consistency across all four tests for each bird, mixed models were used with test round and cue position as fixed effects, and bird ID as a random effect. General linear mixed effect models were used to test for side bias, with reward side and location as fixed effects, and bird ID as a random effect. Gait data residuals were normally distributed, so these data were also analyzed using general linear mixed effect models; gait score was the response variable, enrichment and density were fixed effects, and bird ID nested within pen were random effects. To test for associations between age and gait score, Pearson's chi-square test was used with gait score as the dependent variable and day as an independent variable. Wilcoxon rank-sum test was used to test for links between gait score and latency to approach during the judgement bias test, with latency as the dependent variable and gait score as the independent variable. Data are presented as LSmeans ± SEM unless otherwise noted.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

M.G.A., A.M.C., and L.J. designed and performed the study. M.G.A., A.M.C., and L.J. analyzed the data together with A.C. and G.A. M.G.A and L.J. conceived the first draft of the manuscript and were responsible for managing the data. A.C. and G.A. assisted with revision of the manuscript. All authors read and approved the final manuscript.

Additional information

The authors declare no competing interests.

Chapter 4. Effect of environmental complexity and stocking density on fear and anxiety in broiler chickens

Anderson, M.G.[1], Campbell, A.M.[1], Crump, A.[2], Arnott, G.[3], Newberry, R.C.[4], and Jacobs, L.[1,*]

[1] Virginia Tech, Department of Animal and Poultry Sciences, Blacksburg, VA 24061

[2] London School of Economics and Political Science, Centre for Philosophy of Natural and Social Science

[3] Queen's University Belfast, School of Biological Sciences

[4] Norwegian University of Life Sciences, Department of Animal and Aquacultural Sciences

*Correspondence: jacobsl@vt.edu

4.1 Abstract

Barren housing and high stocking densities may contribute to negative affective states in broiler chickens, reducing their welfare. We investigated the effects of environmental complexity and stocking density on broilers' attention bias (measure of anxiety) and tonic immobility (measure of fear). In Experiment 1, individual birds were tested for attention bias on days 30, 32, and 33 (n=60) and in Experiment 2, groups of 3 birds were tested on days 32, 33, and 38 (n=144). Tonic immobility testing was performed on days 12 and 26 (n=36) in Experiment 1, and on day 19 (n=72) in Experiment 2. In Experiment 1, no differences were observed in the attention bias test.. In Experiment 2, birds from high-complexity pens resumed feeding faster than birds from low-complexity pens following playback of an alarm call, and more birds resumed feeding after playback of a second alarm call, suggesting that birds housed in the complex environment were less anxious. Furthermore, birds housed in high-density or high-complexity pens had shorter

tonic immobility durations on day 12 compared to day 26 in Experiment 1. In Experiment 2, birds from high-density pens had shorter tonic immobility durations than birds housed in low-density pens on day 19, suggesting the former were less fearful at this age. Our results suggest that the complex environment improved welfare of broilers through reduced anxiety.

Keywords: broiler chicken; affective state; environmental complexity; stocking density; anxiety; fear; animal welfare; attention bias; tonic immobility

4.2 Introduction

Environmental enrichment can be defined as "a modification of the environment of captive animals, thereby increasing the animal's behavioral possibilities and leading to improvements of their biological function"[127]. Although results vary depending on the outcome variables assessed, the addition of different structures to the environment adds complexity and can have enriching effects for livestock, including broiler chickens[308,335,336]. These provisions are therefore typically referred to as enrichments.

Fear and anxiety raise welfare concerns because they generate negative affect and, if chronically aroused, highlight an animal's inability to cope with its environment[302,337]. Fear is a short-term emotional response motivating flight from, or freezing in response to, a currently present, immediate threat to survival, while anxiety is a longer-term emotional response motivating vigilance (i.e. alertness) in response to perceived potential threat and is amplified by adverse pre-and postnatal life experiences[337–341]. These systems have evolved as adaptive mechanisms promoting survival in dangerous situations through temporary activation of sympathetic and hypothalamic-pituitary-adrenal axis activity and suspension of growth-promoting parasympathetic actvitiy[337]. However, excessive fear in broilers can be maladaptive, provoking panicked escape behaviors that cause injury, pain, and suffocation[165]. In addition, high levels of fear and anxiety impair the birds' ability to cope with environmental change, such as handling, transport, and loud noises, and have been linked with a worsened feed conversion ratio[310,342]. In many studies, fear in birds is measured using a tonic immobility (TI) test.

TI is an anti-predator freezing response (feigning death) which prey species exhibit as a last resort when captured[343]. Longer TI durations have revealed higher levels of fear in broilers handled roughly compared to gently[344], manually caught compared to mechanically caught[345], or heat-stressed[342] or shocked[346] prior to testing compared to control. A TI test could provide valuable insight into broiler fear levels when handled after rearing in environments varying in complexity and stocking density.

Level of anxiety can be evaluated through an attention bias (AB) test. AB describes the differential, affect-mediated allocation of attention towards one stimulus compared to others[224]. In particular, anxious (vigilance) affective states can increase AB towards a stimulus[224]. Humans with clinical anxiety show a greater AB towards threatening stimuli than those without anxiety[347–349], and studies involving macaques[265], sheep[301,302], cattle[296], and laying hens[297] have validated AB testing as a measure of anxiety level, where animals receiving an anxiogenic drug spent more time looking towards a threatening stimulus and showed increased vigilance behavior compared to control animals. For example, after receiving an anxiogenic drug, laying hens exposed to a conspecific alarm call were slower to feed, faster to vocalize, and exhibited increased locomotion, compared to hens that received a saline injection[297]. These findings suggest that relatively anxious hens allocate more attention to a perceived threat, suggesting that this test could possibly serve as a tool to measure anxiety levels in broilers also. Although studies have reported successful differentiation of AB in animals, others have found unexpected or null results[295,303,350]. To our knowledge, however, AB in broilers has not been previously tested.

Typical broiler chicken housing lacks complexity, such as provision of perches or preferred dustbathing substrate, limiting the expression of diverse natural behaviors, potentially contributing negatively to broiler welfare and performance[127,157,305–307]. High stocking density is another welfare concern in broilers. For instance, high stocking densities can lead to poor foot health[165,336,351] and may increase fear (response to a detected threat)[337]. Lack of environmental complexity has also been associated with fear in broilers[165]. However, behavioral indices of fear were not affected when birds were housed with or without access to string or barrier perches at various stocking

densities[32,51,155], raising questions about how stocking density affects fearfulness of broilers housed in a complex environment.

A reported benefit of adding perches as an enrichment for broilers is that the birds were less aggressive and experienced fewer disturbances while resting compared to broilers without perches[155,157]. For broilers, low perching platforms are used more than single linear perches, probably because heavy birds find them easier to balance on[66], and they were found to reduce avoidance of people, suggesting they reduced fear[352]. Moreover, while broilers are conventionally provided with a single type of litter over the whole floor, adding additional substrate materials can be enriching given that they vary in their value for different functions. For example, sand has been found to increase dustbathing behavior and activity levels compared to rice hull, paper, or wood shaving substrates[69], and adding maize roughage increased foraging behavior compared to wood shavings alone[307]. In addition, broilers housed with novel objects exhibited shorter durations of tonic immobility following acute stressors (sound, heat, and crating stress) compared to the control (no added objects), indicating decreased fearfulness[166]. Given this evidence, increasing environmental complexity with perches, sand, and novel objects would enhance broiler welfare through reduced anxiety and fearfulness.

Potential combined effects of environmental complexity and stocking density on fear and anxiety in broilers have not previously been examined experimentally. Our objective was to investigate the impact of complex housing conditions and stocking density on fearfulness, as measured through a TI test, and anxiety, using an AB test. We hypothesized that broilers housed in a high-complexity, low-density environment would experience the lowest levels of fear and anxiety, whereas broilers from a low-complexity, high-density environment would experience the highest levels of fear and anxiety, with a low-complexity, low-density environment and a high-complexity, high-density environment showing intermediate results. In particular, we predicted that higher levels of fear and anxiety would be reflected by longer TI durations and stronger AB to perceived threatening stimuli.

4.3 Results

4.3.1 Experiment 1 – Attention bias test

Out of the 60 birds tested, 10 birds (4 from LC/LD, 3 from HC/HD, and 3 from HC/LD) began feeding after the first alarm call was played. No differences in latencies to begin feeding were found between either complexity ($\chi^2 = 0.915$; $p = 0.339$) or stocking density ($\chi^2 = 1.715$; $p = 0.190$) treatments (Table 4.1). Seven birds (2 from LC/LD, 2 from HC/HD, and 3 from HC/LD) resumed feeding after the second alarm call was played. No differences in latencies to resume feeding were found between either complexity ($F_{1,6} = 0.528$; $p = 0.544$) or stocking density ($F_{1,6} = 0.892$; $p = 0.444$) treatments (Table 4.1). No differences in latency to first vocalization were found between either complexity ($F_{1,59} = 0.169$; $p = 0.691$) or stocking density ($F_{1,59} = 0.554$; $p = 0.476$) treatments (Table 4.1). Latency to step did not differ between either complexity ($F_{1,44} = 0.016$; $p = 0.904$) or stocking density ($F_{1,44} = 1.925$; $p = 0.215$) treatments (Table 4.1). Looking around tended to be observed more frequently for birds from LD pens compared to birds from HD pens ($\chi^2 = 3.298$; $p = 0.069$; Table 4.2), with no other differences in frequency of observed individual vigilance behaviors between treatments. Vigilance behavior scores did not differ between either complexity ($F_{1,59} = 0.062$; $p = 0.809$) or stocking density ($F_{1,59} = 1.552$; $p = 0.244$) treatments (Table 4.2).

Table 4.1. Least squares mean estimates (s ± SEM) for latency to first vocalization (n = 60), first step (n = 45), begin feeding (n = 60), and raw means (s ± SEM) for latency to resume feeding (n = 7) for broiler chickens kept in high-complexity (HC), low-complexity (LC), high-density (HD), and low-density (LD) treatments in Experiment 1 on days 30, 32, and 33 of age.

Latencies (s)	**Complexity treatment**		**Stocking density treatment**	
	HC	LC	HD	LD
First vocalization	16.40±5.35	19.51±5.35	20.77±5.35	15.14±5.35
First step	39.21±9.58	37.09±13.81	49.21±10.62	27.09±12.46
Begin feeding	265.28±13.95	296.80±1.66	287.69±7.13	274.39±12.67
Resume feeding	56.39±42.24	22.73±20.44	16.22±9.87	58.99±42.03

Table 4.2. Least squares mean estimates (± SEM) for vigilant behavior scores and % of total observations that each type of vigilant behavior was observed for broiler chickens kept in high-complexity (HC), low-complexity (LC), high-density (HD), and low-density (LD) treatments (n = 60) in Experiment 1 on days 30, 32, and 33 of age. Birds were scored either 0 (not observed) or 1 (observed) for each of four vigilant behavior characteristics (erect posture, neck stretching, looking around, and freezing), giving a vigilance score between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed).

	Complexity treatment		**Stocking density treatment**	
	HC	LC	HD	LD
Vigilant behavior score (0-4)	2.53±0.19	2.47±0.19	2.33±0.19	2.67±0.19
Erect posture (%)	43.33	30.00	36.67	36.67
Neck stretching (%)	50.00	53.33	46.67	56.67
Looking around (%)	76.67	46.67	66.67^{B}	86.67^{A}
Freezing (%)	83.33	56.67	83.33	86.67

$^{A-B}$ Proportions with different superscripts differ at $p < 0.1$.

4.3.2 Experiment 1 – Tonic immobility test

An interaction effect of environmental complexity and age was found for TI durations ($F_{1,35} = 6.264$; $p = 0.015$), with longer TI durations for birds from HC pens on day 12 compared to day 29 ($p = 0.004$; Table 4.3). No other pairwise differences were found ($p > 0.12$). Stocking density and age tended to impact TI durations ($F_{1,35} = 3.15$; $p = 0.081$), with birds from HD pens showing longer TI durations on day 12 than on day 29 ($p = 0.016$; Table 4.3). No other pairwise differences were found ($p > 0.17$). Attempts to induce TI did not differ on day 12 between either complexity ($F_{1,35} = 1.03$; $p = 0.318$) or stocking density ($F_{1,35} = 0.041$; $p = 0.84$) treatments, or on day 29 between either complexity ($F_{1,35} = 1.287$; $p = 0.265$) or stocking density ($F_{1,35} = 0.463$; $p = 0.501$) treatments.

Table 4.3. Least squares mean estimates for tonic immobility duration (s ± SEM; 0-300s) and induction attempts (1-3) for broiler chickens kept in high-complexity (HC), low-complexity (LC), high-density (HD), and low-density (LD) treatments on in Experiment 1 on days 12 and 29 (n = 36).

	Bird age (day)	Complexity treatment		Stocking density treatment	
		HC	LC	HD	LD
Mean tonic immobility duration (s)	12	109.43 ± 18.65^{A}	51.24 ± 18.65^{AB}	101.42 ± 18.655^{A}	59.25 ± 18.65^{AB}
	29	31.12 ± 18.65^{B}	49.94 ± 18.65^{AB}	34.31 ± 18.65^{B}	46.75 ± 18.65^{AB}
Mean tonic immobility induction attempt (1-3)	12	2.17±0.19	1.89±0.19	2.06±0.19	2.00±0.19
	29	2.39±0.17	2.11±0.17	2.17±0.17	2.33±0.17

$^{A-B}$ Means with uncommon superscripts differ at $p < 0.05$

4.3.3 Experiment 2 – Attention bias test

Out of the 144 birds tested, 92 began feeding following the first alarm call (19 from LC/LD, 21 from LC/HD, 24 from HC/HD, and 28 from HC/LD). Birds from HC pens began feeding faster than birds from LC pens ($F_{1,143} = 4.430$; $p = 0.043$; Figure 4.1). No differences in latency to begin feeding were found between stocking density treatments ($F_{1,143} = 0.081$; $p = 0.777$). Seventy-eight birds resumed feeding after the second alarm call was played (13 from LC/LD, 15 from LC/HD, 22 from HC/HD, and 28 from HC/LD). No differences in latency to resume feeding were found between either complexity ($F_{1,77} = 2.658$; $p = 0.149$) or stocking density ($F_{1,77} = 2.413$; $p = 0.182$) treatments (Figure 4.1). More birds from HC pens resumed feeding than birds from LC pens (50 from HC, 28 from LC; $\chi^2 = 4.863$; $p = 0.027$). No differences between stocking density treatments were found ($\chi^2 = 2.109$; $p = 0.146$; Figure 4.1). No differences in latency to first step were found between either complexity ($F_{1,99} = 0.005$; $p = 0.946$) or stocking density ($F_{1,99} = 0.834$; $p = 0.368$) treatments (HC: 101.55±20.89s; LC: 101.01±20.82s; HD: 114.51±20.89s; LD:

88.05±20.82s). Neck stretching behavior was observed more frequently in birds from LD pens than HD pens ($\chi^2 = 4.559$; $p = 0.033$), with no other differences in frequency of observed vigilance behavior between treatments. Vigilance behaviors scores did not differ between either complexity ($F_{1,98} = 0.079$; $p = 0.780$) or stocking density ($F_{1,98} = 1.233$; $p = 0.275$) treatment (Table 4.4).

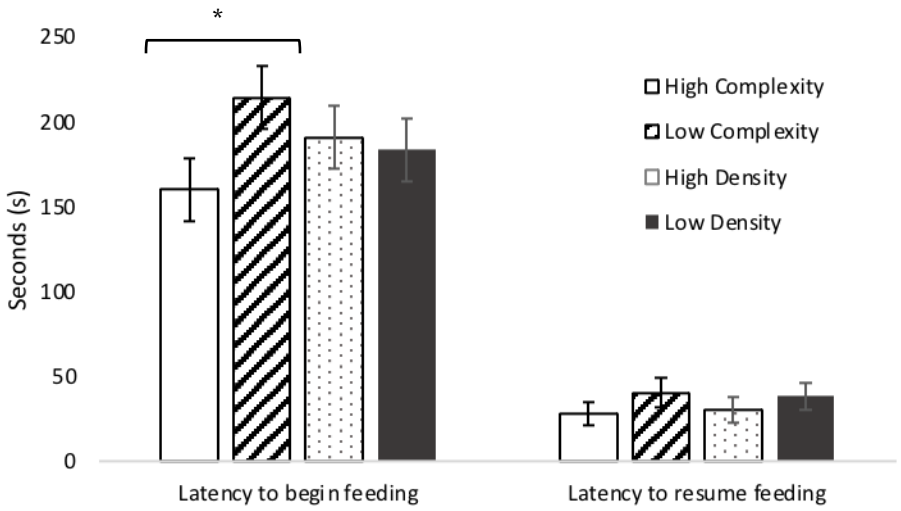

Figure 4.1. Least squares mean estimates (s) for latency to begin feeding (n = 144) and resume feeding (n = 78) for broiler chickens kept in high-complexity, low-complexity, high-density, and low-density treatments in Experiment 2 on days 32, 33, and 38 of age. * $p < 0.05$

Table 4.4. Least squares mean estimates (± SEM) for vigilant behavior scores and % of each type of vigilant behavior observed for broiler chickens kept in high-complexity (HC), low-complexity (LC), high-density (HD), and low-density (LD) treatments (n = 99) in Experiment 2 on days 32, 33, and 38 of age. Birds were scored either 0 (not observed) or 1 (observed) for each of four vigilant behavior characteristics (erect posture, neck stretching, freezing, and looking around), giving a vigilance score between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed).

	Complexity treatment		**Stocking density treatment**	
	HC	LC	HD	LD
Vigilance behavior score (0-4)	2.72±0.15	2.66±0.15	2.57±0.15	2.80±0.15
Erect poster (%)	52.08	45.10	48.98	48.00
Neck stretching (%)	66.67	56.87	51.02b	72.00a
Freezing (%)	62.50	76.47	69.34	70.00
Looking around (%)	89.58	88.24	87.76	90.00

[a-b] Proportions with uncommon superscripts differ at $p < 0.05$.

4.3.4 Experiment 2 – Tonic immobility test

There was no difference in TI duration between complexity treatments ($F_{1,70} = 0.091$; $p = 0.770$). Birds from HD pens had shorter TI durations than birds from LD pens ($F_{1,70} = 12.610$; $p = 0.006$; Figure 4.2). No differences in attempts to induce TI were found between either complexity ($F_{1,70} = 1.016$; $p = 0.341$) or stocking density ($F_{1,70} = 0.074$; $p = 0.793$) treatments. Mean TI induction attempts were 2.08 for HC, 1.86 for LC, 2.00 for HD, and 1.94 for LD pens (SEM of 0.15).

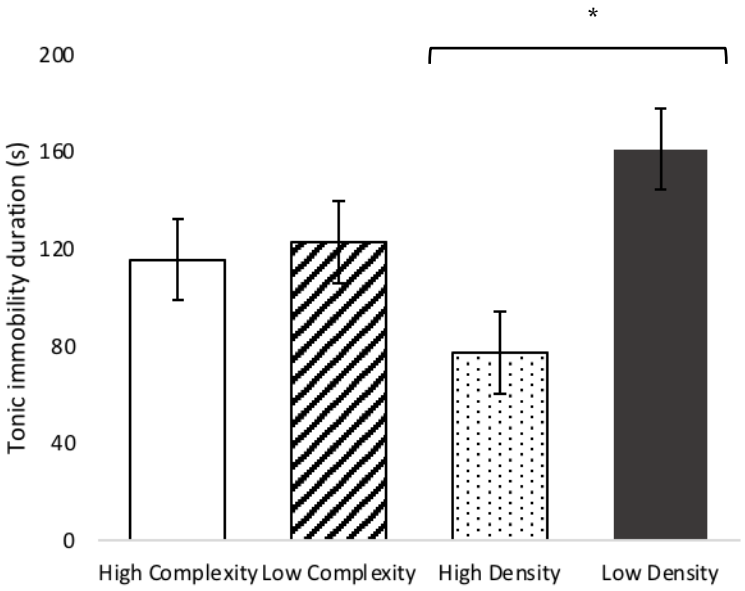

Figure 4.2. Least squares mean estimates (s) for tonic immobility duration (0-300s) for broiler chickens (n = 71) kept in high-complexity, low-complexity, high-density, and low-density treatments in Experiment 2 on day 19 of age. * $p < 0.05$

4.4 Discussion

This study investigated fear and anxiety in broiler chickens housed in either high or low environmental complexities and stocking densities. During the AB test in Experiment 1, birds from LD pens tended to look around more frequently in birds from HD pens, with no differences between the complexity treatments. Birds from HC and HD pens had longer TI durations on day 12 compared to day 29, whereas there was no difference for LC and LD birds. During the AB test in Experiment 2, birds from HC pens began feeding faster than birds from LC pens following the first alarm call playback, more birds from HC pens resumed feeding than birds from LC pens following the second alarm call playback, and birds from LD pens stretched their necks more frequently than birds from HD pens. These results suggest reduced anxiety in birds from HC pens compared to LC pens. Furthermore, birds from HD pens had shorter TI durations than birds from LD pens, indicating reduced fearfulness in birds from HD pens compared to LD pens.

For the AB test, environmental complexity impacted latencies to begin feeding in Experiment 2, but not in Experiment 1. Longer latencies to begin feeding during a threatening situation suggests greater attention allocated towards the threat (alarm call), which indicates a higher level of anxiety. In Experiment 2, birds from HC pens were faster to begin feeding following an alarm call playback than birds from LC pens. This finding suggested reduced anxiousness in broilers housed in complex environments, which was in line with our hypothesis. Conversely, our results suggest that broilers housed in low-complexity environments biased their attention towards a perceived threat compared to a reward (feed). Therefore, these results link low-complexity environments to greater anxiety in broilers. By alleviating these negative states, high-complexity environments appear to improve broiler welfare. Our findings are in line with previous work on starlings[353] and laying hens[298]. Laying hens that preferred to remain indoors during the day responded more anxiously in an AB test compared to hens that preferred to go outside, observed through a small number of indoor-preferring hens eating during the test (only 7% of indoor-preferring hens resumed feeding after the alarm call playback compared to 36% of outdoor-preferring hens)[298]. Latencies to begin feeding in that study were comparable to those in the present study (indoor hens = 160s vs. outdoor hens = 85s compared to broilers from HC pens = 160s vs. birds from LC pens = 214s). Ultimately, our AB results indicate that broilers housed in a complex environment are less anxious than those housed in a low-complexity environment.

Contrary to our predictions, stocking density did not affect birds' responses during the AB test. In line with this finding, one previous study suggested that housing conditions impact broiler welfare more than stocking density[55]. Stocking density can be especially influential later in life, with broiler welfare compromised when stocking densities are higher than 34-38kg/m^2, depending on final body weights[47]. Therefore, the potential detrimental effect of high stocking density could have been absent at the age that AB testing as performed, with high densities ranging between 19-21kg/m^2 (days 30, 32, and 33) in Experiment 1 and 25-30kg/m^2 (days 32, 33, and 38) in Experiment 2. We recommend that future research investigating the effect of stocking density on AB in broilers should perform the test later in life, when densities are at least 34kg/m^2.

The AB test was modified after Experiment 1 to increase sample size and apply a group approach (3 birds tested simultaneously) rather than testing individual birds. Treatments did not impact latency to first vocalization in Experiment 1, latency to begin or resume feeding in Experiment 1, or vigilance behavior scores and latency to first step in both experiments. However, a large numeric difference between latencies to first step in Experiment 1 and 2 was found, with shorter latencies in Experiment 1 (27-49s vs. 88-114s). Broilers in Experiment 1 might have attempted to escape the testing arena faster due to social isolation, while in Experiment 2, broilers experienced social support from flock mates present, reducing their motivation to escape. In line, anecdotal observations did suggest social isolation distress based on the volume and pitch frequency of bird vocalizations and attempts to jump over arena walls in Experiment 1, but not 2. Broilers have a strong motivation for social reinstatement and chickens in natural settings live in relatively small, highly social groups[354–357]. Additionally, pairs of chicks placed in a novel open field test exhibited less fear-related behaviors than individual chicks in the same test[358]. Therefore, latency to first step in a novel testing arena may indicate the birds' motivation for social reinstatement rather than a measure of anxiousness.

The effects of environmental complexity and stocking density on attention bias in broiler chickens were previously unknown. AB tests were pharmacologically validated in laying hens – hens given anxiogenic drugs were slower to feed and faster to vocalize than hens receiving a saline injection, suggesting increased anxiousness in the former[297]. In our study, broilers' latency to first vocalization (15-20s) was much shorter than reported for laying hens, which vocalized after 114s (control) and 317s (hens that received an anxiogenic drug in Experiment 2[297]). Similarly, latencies to first step in broilers was much shorter than (27-114s) or comparable to previously reported results for laying hens (between 42-52s and between 211-355s[297]). Disparities in AB between broilers and laying hens could be due different ages at the time of AB testing or genetic strain differences associated with selection for production traits[359–361]. Broilers have been genetically selected for fast growth rate[362], while laying hens were selected for traits associated with increased egg production[363]. Generally, it is accepted that different strains and breeds of domestic fowl possess different temperaments, most apparent in terms of fear or flightiness, which can be defined as rapid movement away from a

stimulus[364–367]. Therefore, the temperamental differences between broilers and laying hens could explain the difference in responses seen in the AB test.

Environmental complexity can decrease fear in broiler chickens, although some previous studies found no relationship. Access to elevated platforms resulted in shorter TI durations (238s vs. 311s) compared to access to manipulated standard resources (greater distance between feeders and water lines), suggesting reduced fearfulness in broilers housed with platforms[368]. These TI durations are longer than those observed in the current study, even though test approaches were comparable (LC: 123s vs. HC: 116s in Experiment 1). Broilers housed with perches and dust baths had shorter flight distances in an avoidance test, suggesting they were less fearful towards humans than control birds[28]. In Experiment 1, we found a difference in fearfulness within complexity treatments at different ages, but found no difference between complexity treatments. This agrees with other studies that did not report an impact of complexity on fear. For example, broilers housed with barrier perches did not have different TI durations compared to control birds[155,169]. Furthermore, responses during a novel object test to assess fearfulness did not differ between broilers housed with or without string enrichments[51]. Our results indicate that providing multiple enrichments concurrently does not impact fearfulness in broilers.

We hypothesized that birds from HD pens would have longer TI durations and require fewer attempts to induce TI than birds from LD pens, indicating greater fear. However, in Experiment 1, we did not establish a difference between treatments on TI durations, only an interaction within HC and HD treatments with age. In Experiment 2, we found that birds in LD pens had longer TI durations than birds in HD pens (LD: 161s versus HD: 72s in Experiment 2). Past research suggests housing broilers at high stocking densities can contribute to increased fearfulness, which is contrary to our result. For example, broilers housed at a density of more than 18 to 22 birds/m^2 had longer TI durations than broilers housed at lower densities[351,369,370]. Another study found that broilers housed at a high stocking density of 56kg/m^2 showed longer TI durations (more fearful) than broilers housed at lower densities[351]. Two of these lower stocking densities were comparable to the high and low densities at the time of TI testing in our study

(6kg/m^2 and 15kg/m^2 compared to 8-16kg/m^2 and 4-8kg/m^2 in the present study), yet they did not find differences in TI duration between those two density levels (112s for birds housed at 6kg/m^2 versus 101s for birds housed at 15kg/m^2). The difference in results could be attributed to an age effect, as birds were tested for TI at week 6 of age in the previous study compared to weeks 2 and 3 of age in the present study, possibly indicating broilers are more fearful early in life. Young, small birds may perceive "safety in numbers" of greater importance at a low density than older, large birds. This hypothesis is supported by our results from Experiment 1, where birds from HD and HC pens had longer TI durations on day 12 compared to day 26, suggesting they were more fearful early in life. Nevertheless, this decrease in TI duration with age could reflect habituation to the test and handling, as the same birds were tested on both days. Contrary to our predictions and previous findings, birds from HD pens were less fearful than birds from LD pens. We recommend further research on this relationship.

We investigated the effects of housing broiler chickens in a high- or low-complexity environment under high or low stocking densities on their level of fear and anxiety. The group approach to AB testing in Experiment 2 produced a difference in broiler responses between the complexity treatments, compared to the individual testing approach in Experiment 1. Broilers from high-complexity pens exhibited responses in the AB test suggestive of reduced anxiety compared to broilers from low-complexity pens, with no differences between the stocking density treatments. These results suggest that the environmental conditions (complexity) provided in the present study elicited a stronger impact on anxiety than stocking density. To our knowledge, this is the first AB test validated to assess anxiety and welfare in broiler chickens. Additionally, birds housed at higher stocking densities reduced TI duration, suggesting reduced fearfulness compared to birds housed at lower stocking densities. This finding counterintuitively indicates that, for broilers around 3 weeks old, housing at higher densities may improve welfare.

4.5 Materials and Methods

Birds, housing, and treatments

Two experiments were conducted. In each, 1,620 male Ross 708 chicks (total n = 3,240), vaccinated against Marek's disease, were obtained at day 0 from a commercial hatchery (PA, USA). Upon arrival to the research facility, chicks were randomly allocated to one of four treatment groups in a 2×2 factorial design with environmental complexity (low-complexity [LC] vs high-complexity [HC]) and stocking density (low-density [LD] vs high-density [HD]) as factors at pen level. Each treatment group was replicated three times (12 pens in total), distributed in a randomized complete block design.

All pens (14.5m^2) contained standard pine shavings as bedding (approximately 10cm depth), four round hanging tube feeders, and three water lines, each with three nipple drinkers. All birds had ad libitum access to water and commercial broiler chicken feed (starter day 0-14, grower day 15-28, and finisher day 29-50). Birds had access to three heat lamps/pen and 24h light in the first 7 days, followed by a light:dark schedule of 18L:6D, with a light intensity of approximately 15 lux during light hours. Due to a technical issue in Experiment 1, birds received 24h light for 7 additional days during week 2 of age. House temperature was gradually decreased from 35°C on day 1 to 21°C on day 50 by assessing bird comfort. In Experiment 1, all birds received a therapeutic dose of antibiotics via the water lines from day 33-40 in response to a pathogen exposure.

Environmental enrichment

HC pens contained four functional spaces (Figure 4.3a), including space for 'feeding' (approximately 3m^2), 'comfort' (approximately 3m^2), 'resting' (approximately 3m^2), and 'exploration' (approximately 4.3m^2). The feeding, comfort, and resting spaces included a water line. The feeding space contained four feeders and one third of a medium PECKstone™ (Proteka, VILOFLOSS, Germany) broken into smaller pieces. The comfort space contained a wooden-frame dust bath (180cm L × 91cm W × 10cm H) filled with 68kg of playground sand (QUIKRETE, GA, USA) that was raked and partially replaced

when depleted. The resting space in Experiment 1 included three perches (182.9cm L × 30.5cm W × 8.5cm H) constructed of 1.91cm diameter PVC pipe, which was sprayed with textured black spray paint (Rust-Oleum, IL, USA) to enhance grip while perching (Figure 4.4a). Birds had access to 7.6cm of linear perch space/bird in high-density pens, and 15.2cm/bird in low-density pens. In Experiment 2, the PVC pipes were replaced with three wide wooden perches forming a platform (121.9cm L × 45.7cm W × 7.6cm H; Figure 4.4b), providing 76cm^2 of space/bird in the low-density pens, and 39cm^2 of space/bird in the high-density pens. The exploration space contained a pair of enrichment objects, starting on day 2 of age. Six objects were randomly paired into three groups of two, combining a nutritional and an occupational enrichment object, and these pairs were rotated every three days according to a randomized schedule to maintain variation and novelty (Table 4.5). The LC pens had a similar set-up to the HC pens with four spaces, but without the peck stones, dustbath, perching platforms, or enrichment objects to differentiate the spaces into different functional areas (Figure 4.3b).

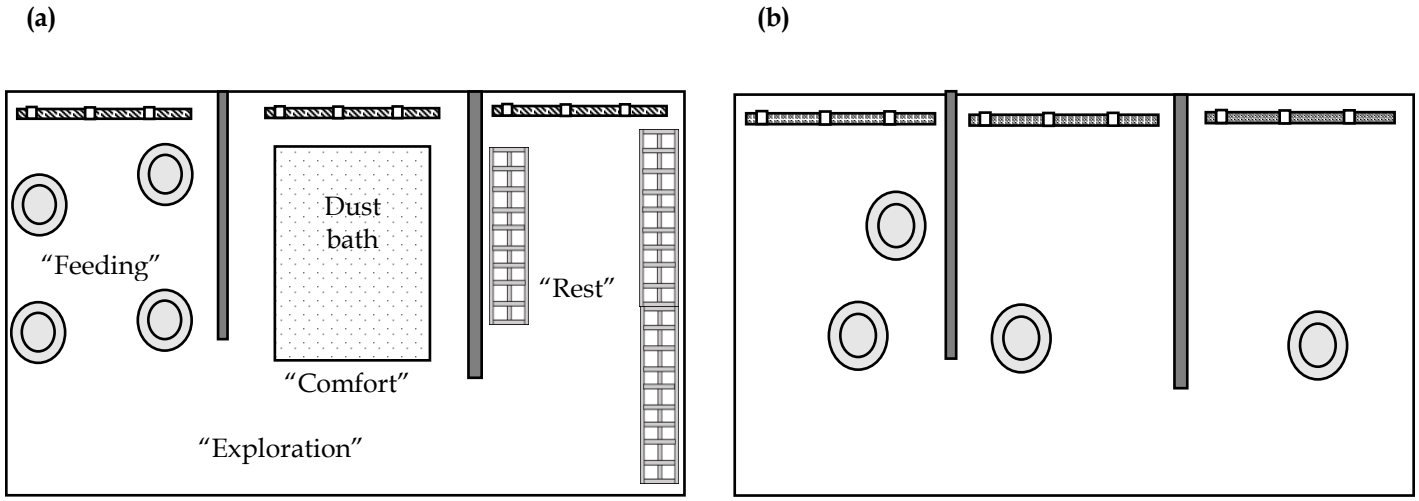

Figure 4.3. (**a**) High-complexity pen with four functional spaces for 'feeding', 'comfort', 'resting', and 'exploration'. The feeding space contained four feeders (○) and pecking stones, the 'comfort' space included a sand dust bath (□), the resting space contained three perches (▭), and the exploration space contained varying pairs of enrichment objects. The feeding, comfort, and resting spaces each contained a water line with three nipple drinkers (▬). (**b**) Low-complexity (control) pen, containing four feeders and three water lines.

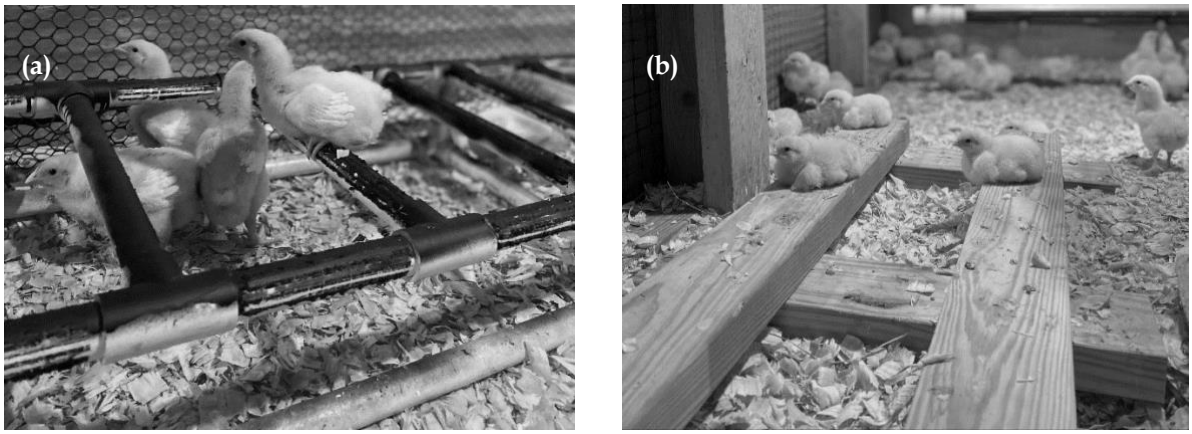

Figure 4.4. Photograph of the perch design in high-complexity pens in (**a**) Experiment 1 (n = 3/pen) and (**b**) Experiment 2 (n = 3/pen).

Table 4.5. Pairs of enrichment objects (toys) rotated every 3 days in high enrichment (HE) pens.

Nutritional enrichment	n	Occupational enrichment	n	Start day of rotation (day)					
Hanging bundles of white string	8	Free-moving metal ball (20.3cm diameter)[5] filled with alfalfa hay	4	2	11	20	29	38	47
Yellow treat dispenser (7.6cm diameter)[1] filled with whole-grain oats	4	Colored ball (5.8cm diameter)[2]	4	5	14	23	32	41	
Laser light[3]	5 min 2/day	Experiment 1: Kong toy (5.6cm diameter)[4] filled with iceberg lettuce	4	8	17	26	35	44	
		Experiment 2: half a head of cabbage hung at bird height	1						

[1] Lixit Corp., CA, USA

[2] Click N' Play, USA

[3] Ethical Products, Inc., NJ, USA

[4] KONG, CO, USA

[5] Darice, OH, USA

Stocking density

The HD pens were stocked with 180 chicks/pen, resulting in 42.1kg/m^2 at day 50 in Experiment 1, and 42.6kg/m^2 in Experiment 2 (Table 2). The LD pens were stocked with 90 chicks/pen and reached a density of 23.8kg/m^2 at day 50 in Experiment 1, and 23.3kg/m^2 in Experiment 2 (Table 4.6).

Table 4.6. Mean pen stocking densities at day 1, 29, and 50 in kg/m² and birds/m² in Experiments 1 and 2.

Stocking density	Experiment 1					
	Day 1		Day 29		Day 50	
	Kg/m²	Birds/m²	Kg/m²	Birds/m²	Kg/m²	Birds/m²
High	0.52	13.85	18.93	13.14	42.08	12.31
Low	0.26	6.92	9.81	6.71	23.83	6.29
Stocking density	**Experiment 2**					
	Day 1		Day 29		Day 50	
	Kg/m²	Birds/m²	Kg/m²	Birds/m²	Kg/m²	Birds/m²
High	0.46	12.41	19.90	12.23	42.64	11.56
Low	0.23	6.21	10.22	5.97	23.31	5.79

4.5.1 Experiment 1 – Attention bias test

A square testing arena was constructed with two plastic, perforated folding partitions (approximately 124.5cm L × 124.5cm W × 91.4cm H) with pine shavings on the floor and a feeder containing commercial feed, oats, and mealworms (Figure 4.6). The arena was located in a separate room adjacent to, but separate from, the broilers' home pens.

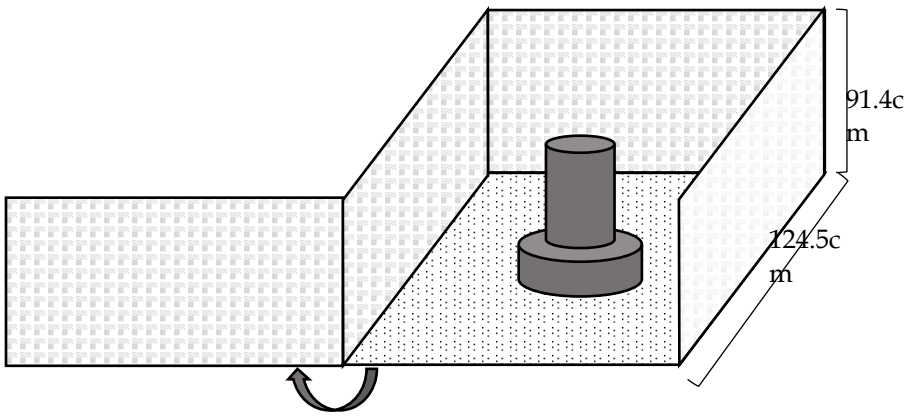

Figure 4.6. Diagram of the attention bias (AB) arena used in Experiments 1 and 2. A familiar feeder was placed in the center of the arena and wood shavings were provided as litter.

AB testing (modified from[224,297,371]) was performed with five randomly selected birds/pen (n = 60) on days 30, 32, and 33 of age. The testing order of pens was randomized. Each bird was tested separately by two observers. The test started when the bird was placed in the AB arena. Immediately thereafter, an 8 second (s) conspecific alarm call was played from portable speakers (FUGOO, Van Nuys, CA, USA) at full volume (95dB). The alarm call was recorded from a chicken signaling a ground predator, which previous playback experiments have found to elicit a vigilance response[371]. Following the alarm call, latency to begin feeding was recorded. If the bird began feeding at any point during the test, it was allowed approximately 10s to feed, then the alarm call was played a second time, and latency to resume feeding was recorded. The test ended when the bird resumed feeding a second time (maximum test duration of 300s). Birds that never began feeding received a maximum latency to begin feeding score of 300s and those failing to resume feeding received no score. Additional live-recorded variables included latency to first vocalization and occurrence (yes/no) of vigilance behaviors in the 30s following the first alarm call (visibly stretching neck, looking around, freezing, and erect posture)[297]. Each of the four vigilance behavior characteristics (erect posture, neck stretching, looking around, and freezing) were scored

as either 0 (not observed) or 1 (observed), giving a vigilance score between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed at least once) for each bird tested. Videos were used to record latency to first step from when the alarm call playback ended, as a potential additional indicator of anxiety to determine how long the birds remained in a motionless state after the alarm call playback[297,298].

4.5.2 Experiment 2 – Attention bias test

After Experiment 1, the AB test was modified with an increased sample size, a group testing approach rather than testing individual birds, and allowing more time in the test arena if most (but not all) birds began feeding after the first alarm call was played. The AB test was performed on days 32, 33, and 38 of age with 12 randomly selected birds/pen (n = 144) by two observers. The order of pens was randomized for testing. Birds were tested in groups of 3 (4 tests/pen) to avoid isolation stress[358]. The same location, arena, feeder, feed, and alarm call were used as described for Experiment 1 (Figure 4.6). Immediately after 3 birds were placed into the arena, the 8s conspecific alarm call was played. Latency to begin feeding (s) from the feeder was then recorded for each individual bird. Thereafter, the test procedure had four possible outcomes depending on how many birds began feeding and the time-point that they started feeding within the first 300s of the test.

- If all 3 birds fed from the feeder at least once during the 300s testing period, they were allowed 5s to feed before the second alarm call playback. Thereafter, the second alarm call was played. If all 3 birds fed from the feeder between 270-300s, birds were allowed to feed for 5s starting from when the last bird fed, the second alarm call was played, and the test time was extended to 420s. Latency to resume feeding was recorded for each individual bird.

- If at the end of the 300s testing period, 2 out of 3 birds fed from the feeder, they were allowed 5s to feed starting from when the last bird fed, then the second alarm call was played and the testing time was extended to 420s. Latency to resume feeding was recorded for each individual bird. The bird that did not feed

received a maximum latency score of 300s for latency to begin feeding and no score for latency to resume feeding.

- If 1 of the 3 birds fed from the feeder during the testing period, latency to begin feeding was recorded for the bird that began feeding, and the second alarm call was not played. The other 2 birds received a maximum latency score of 300s.

- If none of the 3 birds fed from the feeder during the testing period, all 3 birds received a maximum latency score of 300s.

Video recordings were also used to record latency to step (s) and occurrence (yes/no) of vigilant behaviors within 30s following the first alarm call. Each of the four vigilance behavior characteristics (erect posture, neck stretching, looking around, and freezing) were scored as either 0 (not observed) or 1 (observed), giving a vigilance score between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed at least once) for each bird tested. It was not feasible to record latency to first vocalization because birds were tested in groups.

4.5.3 Tonic immobility test

In both experiments, a single observer performed TI testing in the hallway area of the house, directly adjacent to the birds' home pens. In Experiment 1, TI testing was performed on 3 randomly-marked birds/pen (n = 36) on day 12 of age. The same marked birds were tested again on day 26 of age. In Experiment 2, TI testing was performed on 6 randomly selected birds/pen (n = 72) on day 19 of age. TI was induced by the handler carefully placing the bird on his back in a V-shaped cradle, placing one hand over the sternum and applying gentle pressure while cupping the other hand over the head (modified from[372]).. After 15s, the handler lifted her hands from the bird, moved out of the bird's line of sight, and recorded latency until righting response (TI duration [s]). If the bird attempted to right himself within 10s after the hands were lifted, TI was considered not induced and the handler repeated the restraint procedure (maximum of 3 induction attempts). If TI could not be induced, the bird received the minimum score of 0s. If birds remained in TI for the full 300s testing period, a maximum latency score of 300s was given.

Statistical analysis

Data were analyzed in JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). Data residuals were assessed for their distribution by visual inspection of normal quantile plots. An overview of the distribution of data residuals and subsequent statistical approaches is shown in Table 4.7. The resumption of feeding in the Experiment 1 AB test was too low for statistical analysis, so raw means are presented. For normally distributed data (see Table 4.7), with the exception of AB data in Experiment 2, general linear mixed-effects models were used, with complexity (HC/LC), stocking density (HD/LD), and their interaction as fixed effects, and pen as a random factor. Normally distributed AB data in Experiment 2 were analyzed using general linear mixed-effects models, with complexity (HC/LC), stocking density (HD/LD), and their interaction as fixed effects, and testing group nested within pen as a random factor. No significant interaction effect was found for any response variables, so the interaction term was removed from the models. Durations of TI in Experiment 1 were analyzed using general linear mixed-effects models with complexity (HC/LC), stocking density (HD/LD), and day (bird age) as fixed effects, with pen as a random factor. Tukey's HSD test was used for post-hoc analysis. Occurrence of vigilance behaviors were summed to give a total score, which ranged between 0 (no vigilance behavior observed) and 4 (all vigilance behaviors observed at least once), then were analyzed with complexity and stocking density as fixed effects, and pen as a random factor. Data are presented as LSmeans ± SEM unless otherwise noted.

Table 4.7. Summary of data analyses for Experiments 1 and 2.

Test	Response variable (unit)	Distribution of data residuals	Statistical approach
Attention bias	Latency to first vocalization (s)[1]	Normal	General linear mixed-effects model
	Latency to first step (s)	Normal	General linear mixed-effects model
	Latency to begin feeding (s)	Other	Chi-square[1] and general

			linear mixed-effects model[2]
	Latency to resume feeding	Normal	General linear mixed-effects model
	Frequency to resume feeding (% of tested birds)[2]	Other	Chi-square
	Vigilant behavior scores (0-4)	Normal	General linear mixed-effects model
	Frequency of vigilance behaviors	Other	Chi-square
Tonic immobility	Duration (s)	Normal	General linear mixed-effects model

[1]In Experiment 1

[2]In Experiment 2

Author Contributions: Conceptualization and methodology, M.G.A., A.M.C., R.C.N., and L.J.; formal analysis and data curation, M.G.A. and L.J.; writing – original draft preparation, M.G.A. and L.J.; writing, review, and editing, M.G.A., A.M.C., A.C., G.A., R.C.N., and L.J.; project administration, M.G.A., A.M.C., and L.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Both experiments in this study were approved by Virginia Tech's Institutional Animal Care and Use Committee (approval #19-175). The study was conducted in accordance with the IACUC's guidelines.

Data Availability Statement: Data are available upon request from the corresponding author.

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Chapter 5. Impact of environmental complexity and stocking density on affective states of rainbow trout (*Oncorhynchus mykiss*)

Anderson, M.G. [1], Campbell, A.M. [1], Kuhn, D.D.[2], Smith, S.A.[3], and Jacobs, L.[1*]

[1]Virginia Tech, Department of Animal and Poultry Sciences, Blacksburg, VA, USA

[2]Virginia Tech, Department of Food Science and Technology, Blacksburg, VA, USA

[3]Virginia-Maryland College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology, VA, USA

*Corresponding author: jacobsl@vt.edu, +1 540-231-4745, 175 West Campus Drive, Blacksburg, VA, 24061

5.1 Abstract

Affective states of animals can be assessed through judgement bias tests, evaluating responses to ambiguous situations. In this study, rainbow trout (n = 108) were housed in recirculating aquaculture systems under commercial conditions while trained at tank-level to discriminate between a putative reward (feed) in one chamber and a putative aversive (chase by net for 1s) in the opposing chamber. Fish from successful tanks (2 out of 5 tanks) were then housed in treatment tanks of either high or low environmental complexity at either high (165 fish/m^3) or low (69 fish/m^3) stocking density. Trained fish were tested for latencies to approach three intermediate, ambiguous chambers. Fish housed in high-density tanks were faster to enter all chambers than those housed in low-density tanks (8.5s vs. 15.2s; P = 0.001), with faster entries into the positive (7.4s vs. 15.2s; P = 0.02) and near-negative chambers (10.2s vs. 17.4s; P = 0.006), suggesting that these fish were more optimistic to receive a feed reward. Tank complexity did not affect test outcomes. No differences between treatments were observed between body weight, length, and plasma cortisol. Overall, rainbow trout are capable of discriminating between cues during a judgement bias test and fish housed in high-density

environments respond more optimistically in ambiguous situations compared to fish in low-density environments.

Keywords: animal welfare, rainbow trout, environmental complexity, enrichment, stocking density, affective state

5.2 Introduction

The welfare of terrestrial livestock has undergone much study, partly because of a push from concerned consumers. However, relatively little has been investigated about the welfare of our aquatic food sources. Rainbow trout (*Oncorhynchus mykiss*) are typically housed in a variety of barren ponds, raceways, or recirculating aquaculture systems[373]. These environments provide no access to biologically-relevant enrichments, besides that of interaction with conspecifics. Environmental complexity can have a positive impact on animals' biological functioning and behavior[127,181]. For example, rainbow trout reared in enriched environments with cobblestone tank substrate had better fin condition, and steelhead trout (*Oncorhynchus mykiss irideus*) housed in tanks with unmoved rocks exhibited more exploratory behavior than fish in a barren control environment[187,189]. Stocking density is another environmental aspect to consider, but guidelines differ due to the complex interactions between density, fish behavior, fin damage, availability of feed, and type of rearing system[1,87,94]. High stocking densities could indirectly affect fish welfare through reduced water quality, decreased growth rate, and competition for feed[1,87,119,374]. Therefore, it is important to consider the effect of stocking density, in addition to environmental complexity, on fish health and welfare.

Fish can experience negative emotions, such as pain, fear, and suffering, just like their terrestrial counterparts, through systems similar to the prefrontal cortex[375–377]. These short-term emotions are adaptive and allow the animal to appropriately respond to changing environments[14]. Short-term emotional responses can shape an animal's affective state, which are long-term states that reflect the cumulative valence of emotions over time[13]. Affective states can influence the way an animal makes decisions (cognitive

bias)[211–214,223,276,278,378,379]. Cognitive bias tests are a well-validated indicator of animal welfare for a variety of terrestrial species, however there is little evidence of this type of test being employed in an aquatic setting for farmed fish[14,227,228]. One type of cognitive bias test measures a subject's judgement bias through responses to ambiguous cues, which is then used to determine the subject's level of optimism or pessimism. Shorter latencies to approach ambiguous cues would indicate optimism (greater expectation of a reward), whereas longer latencies to approach ambiguous cues would indicate pessimism (lower expectation of a reward)[13,14,219,222,270]. Ultimately, the judgement bias test has been considered the "gold standard" for evaluating affective states in animals and could be a valid tool to assess fish welfare[323].

Environmental complexity has the potential to positively impact fish welfare and affective states. Providing shelter structures has been shown to decrease aggression, fin erosion, and distress in Atlantic salmon (*Salmo salar*) and rainbow trout, possibly because fish have the opportunity to escape bullies[187,380–382]. Artificial vegetation decreased the frequency of startle responses in Tiger Muskellunge (*Esox masquinongy x Esox lucius)* and reduced the habituation period for bream (*Abramis brama*) in experimental conditions[383,384]. Additionally, providing floating artificial vegetation can serve as partial visual cover, which is preferred over unshaded areas, and can increase growth rate and decrease stress of Atlantic salmon[385,386]. Based on prior findings, providing environmental complexity within the tank could lead to a reduction of emotions associated with a negative affective state and induce an overall positive affective state.

Affective states of zebrafish (*Danio rerio*) housed in different environments were successfully evaluated through a judgement bias test[277]. Zebrafish housed in enriched tanks showed more exploratory behavior within ambiguous cues than those housed in barren tanks, suggesting affective state was manipulated by environmental conditions. Additionally, female cichlid fish (*Cichlidae*) housed with non-preferred males showed pessimistic responses during a judgment bias test[276]. Based on these studies, judgement bias tests could be a useful tool to assess affective states of rainbow trout housed under varying environmental conditions, however this has not yet been employed.

The objective of this study was to evaluate the effects of environmental complexity and stocking density on affective states of rainbow trout through a judgement bias test. Additional measurements were taken, such as individual weight and length to evaluate potential impact of housing environments on production outcomes, feeding behavior, and plasma cortisol to assess fish stress levels. We hypothesized that fish reared in high-complexity tanks of either high- or low-density would exhibit increased optimism in the judgement bias test through shorter latencies to approach ambiguous cues compared to fish reared in low-complexity tanks of either density. We also expected weight and length of fish from high-complexity, low-density tanks to be greater than fish from any other treatment group. We expected fish housed in high-density tanks of either complexity level to show the shortest latencies to begin feeding compared to all other treatment groups because of increased competition for feed. Lastly, it was hypothesized that fish housed in high-complexity, low-density tanks would show decreased stress when compared to all other treatment groups.

5.3 Results

5.3.1 Judgement bias test

After 32 days, fish kept in high-complexity/high-density (HC/HD), high-complexity/low-density (HC/LD), low-complexity/high-density (LC/HD), and low-complexity/low-density (LC/LD) treatments were tested for judgement bias, measuring latencies to enter the positive (POS), negative (NEG), and 3 ambiguous chambers (near positive, NP; middle, MID; near negative, NN). Mean latencies to enter all chambers were 10.91 ± 1.59s for test session 1, 11.94 ± 1.59s for test session 2, and 12.55 ± 1.59s for test session 3. Testing session did not impact responses of fish during the judgement bias test ($F_{1,2}$ = 0.973; P = 0.380).

Environmental complexity did not affect latencies for fish to enter all chambers during the judgement bias test (HC = 11.76 ± 0.95s; LC = 11.84 ± 0.95s; P = 0.955). Furthermore, environmental complexity did not impact latencies to enter the POS ($F_{1,6}$ = 0.02; P = 0.891), NP ($F_{1,6}$ = 0.11; P = 0.752), MID ($F_{1,6}$ = 0.173; P = 0.692), NN ($F_{1,6}$ = 1.026; P = 0.35),

or NEG ($F_{1,6}$ = 0.121; P = 0.739) chambers (Table 5.1). There was no interaction effect of chamber type and complexity level on latencies to enter all chamber cues ($F_{1,148}$ = 0.699; P = 0.594).

Table 5.1. Mean latency for the first fish to enter (s ± standard error) each chamber during the judgement bias test for trout in either high- or low-complexity tanks (n = 8 tanks; 38 fish).

Complexity level	Mean latency (s) for the first fish to enter (LSM±SEM)				
	POS	NP	MID	NN	NEG
High	11.56 ± 1.69	11.17 ± 2.75	10.41 ± 2.76	12.09 ± 2.33	13.39 ± 2.45
Low	11.01 ± 1.69	9.87 ± 2.75	8.78 ± 2.76	15.43 ± 2.33	14.60 ± 2.45

Fish from HD tanks were faster to enter the NN ($F_{1,6}$ = 20.3; P = 0.006) and POS ($F_{1,6}$ = 10.797; P = 0.022) chambers compared to fish from LD tanks (Figure 5.1). Additionally, fish from HD tanks tended to enter the MID ($F_{1,6}$ = 4.68; P = 0.083) and NP ($F_{1,6}$ = 5.075; P = 0.074) chambers faster than fish from LD tanks. There were no pairwise differences in latency to enter the NEG chamber ($F_{1,6}$ = 3.118; P = 0.138; Figure 5.1).

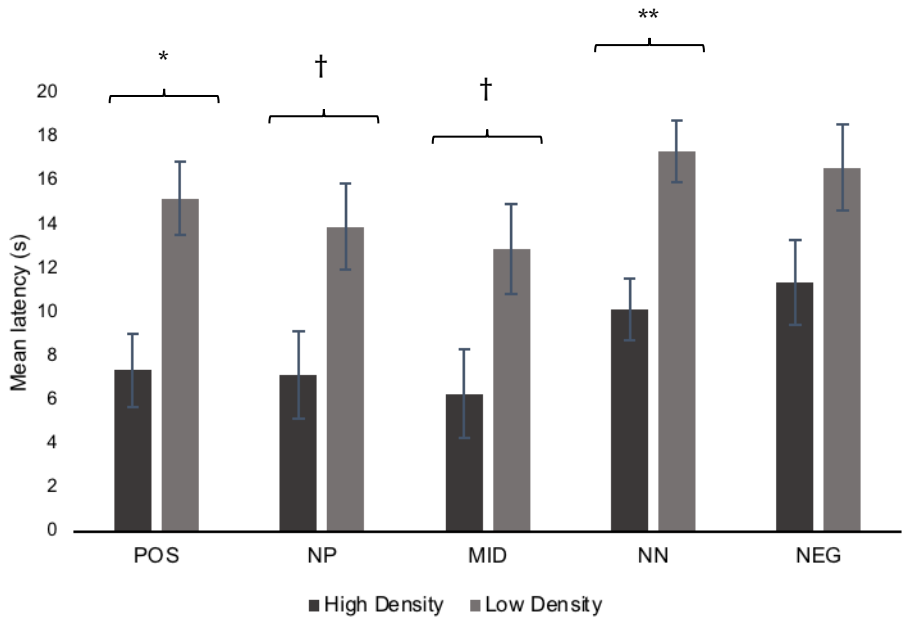

Figure 5.1. Mean latency for the first fish to enter (seconds) each chamber during the judgement bias test for trout housed under high or low stocking density (n = 8 tanks; 38 fish). ** $P < 0.01$; * $P < 0.05$; † $P < 0.1$.

5.3.2 Body weight and standard length

There was no effect of environmental complexity or stocking density on body weight and length of fish on day 64 or 96 ($P > 0.1$; Figure 5.2).

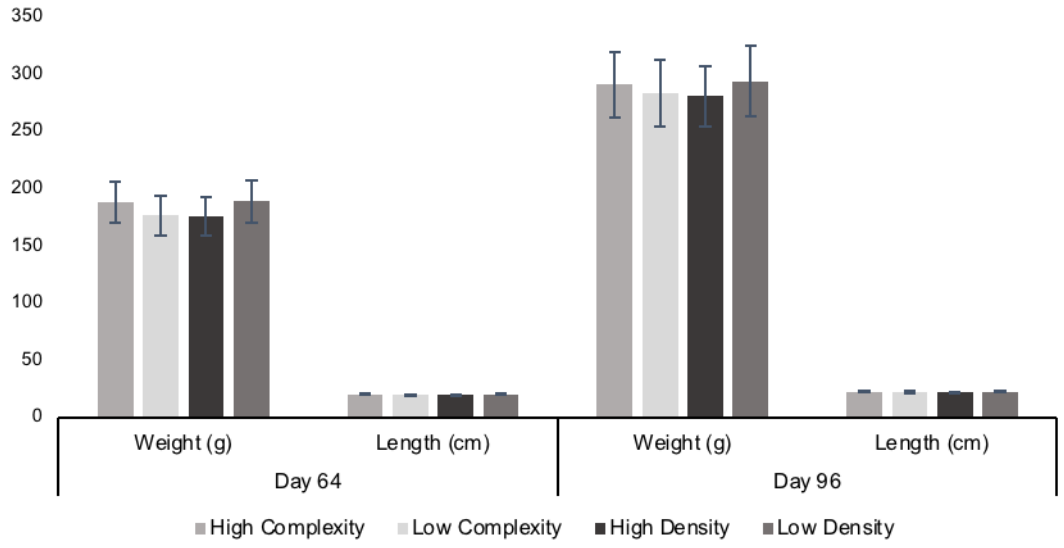

Figure 5.2. Mean weight (g) and length (cm) ± standard error of fish from each treatment group, measured on days 64 (n = 68) and 96 (n = 66).

5.3.3 Feeding behavior

Environmental complexity did not impact latencies to begin feeding during daily feedings ($F_{1,15} = 0.161$; $P = 0.709$; Figure 5.3a). However, fish housed in high-density tanks had shorter latencies to begin feeding than fish housed in low-density tanks ($F_{1,15} = 13.9$; $P = 0.0203$; Figure 5.3b).

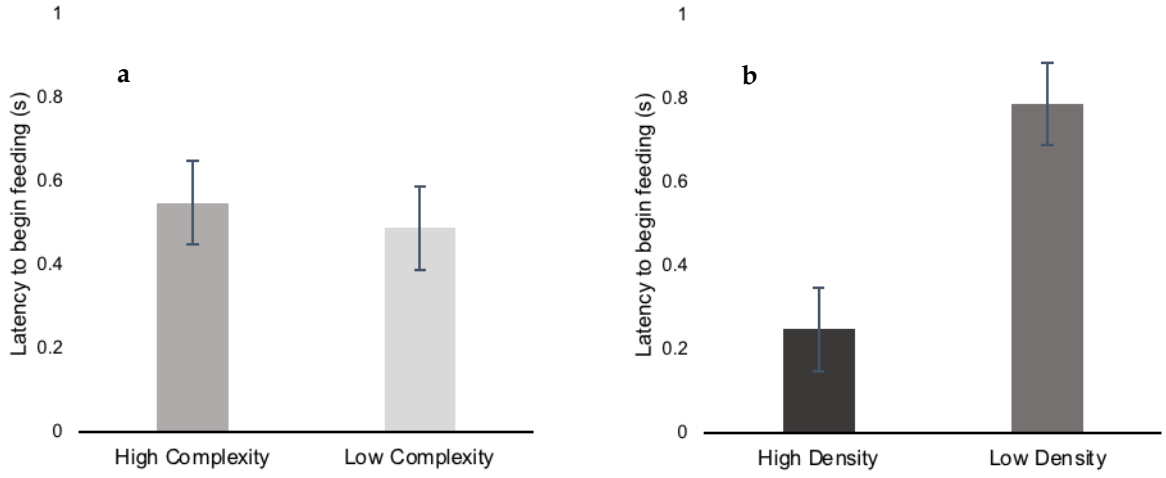

Figure 5.3. Mean latencies for the first fish to begin feeding (seconds) during daily feedings on days 79-95 for fish housed in a) high- or low-complexity tanks or b) high or low stocking density tanks (observation n = 128).

5.3.4 Plasma Cortisol

Cortisol concentrations did not differ between treatment groups ($P > 0.1$). Mean cortisol concentrations were 1.16 ng/mL for HD, 3.68 ng/mL for LD, 3.09 ng/mL for HC, and 1.74 ng/mL for LC fish (standard error = 1.10; n = 34).

5.4 Discussion

This study is the first to apply a judgement bias test to evaluate affective states of rainbow trout. While housed in commercial conditions, fish were trained at tank-level to discriminate between two opposing locations of an arena, with one location associated with a feed reward and the other location associated with being chased by a net. After 61 days of training, two of the five tanks successfully passed the learning criterion to be tested. Then, we attempted to manipulate affective state by placing fish in either high- or low-complexity tanks under either high- or low-density. Fish from HD tanks were faster to enter all chambers than fish from LD tanks, and had shorter latencies to enter the NN

and POS chambers, suggesting optimism for a reward in the fish from HD tanks. Environmental complexity did not affect latencies to enter chambers during the judgement bias test, suggesting environmental complexity had no impact on fish affective state or optimism in this study.

The lack of impact of the environmental complexity treatment was opposite to our hypothesis. We provided environmental enrichments that were biologically relevant and ultimately improved trout affective state through rearing them in an environment closest to that of their natural living conditions[127]. In their natural habitat, rainbow trout will seek cover in the form of overhanging vegetation, undercut banks, aquatic vegetation, logs, or debris piles to rest and avoid predation[81]. We used artificial floating lily pads as visual cover because it has been shown that rainbow trout prefer darker areas of a tank environment over exposure to bright lights, and seek safety in these areas when in the presence of a threatening stimulus[387,388]. Additionally, a PVC shelter structure was provided, because the presence, not necessarily the utilization, of shelters reduced basal cortisol and metabolic rates in Atlantic salmon[382,389]. Finally, artificial cabomba plants were used to simulate aquatic vegetation, a form of shelter used by wild rainbow trout fry[81]. Similar environmental enrichments (shelter and gentle light) for zebrafish resulted in more optimistic responses during the judgement bias test compared to the control, with more exploratory behavior within the ambiguous cues (28% of observed time compared to 7.5%)[277]. Social enrichment for cod (*Gadus morhua*) affected their cognitive bias; cod housed with a larger, more aggressive conspecific for 24 hours were 12 times less likely to enter ambiguous chambers during a judgement bias test compared to fish housed in social isolation for 24 hours[278]. Similarly, female cichlids housed with an unpreferred male showed longer latencies to respond to the ambiguous signal than females housed with a preferred male (approximately 600s versus 300s)[276]. These studies show that judgement bias can be influenced by environmental conditions, however it is possible that fish in our study were not exposed to the enrichments for long enough to observe any effect on affective state, with 3 weeks of exposure prior to judgement bias testing in our study compared to 7-18 weeks in previous work. Alternatively, the effect of density might have overshadowed any potential effect of environmental complexity. Perhaps the LD treatment was too great of a stressor that

environmental complexity could not alleviate that stress. Similarly, the HD environment may have provided such a welfare benefit that environmental complexity could not contribute further to trout responses.

To our knowledge, there are no published studies investigating the effects of stocking density on affective states of trout. During testing, fish from HD tanks were overall more optimistic than fish in LD tanks, and specifically more optimistic to receive a reward in the NN chamber. Fish from HD tanks also tended to be more optimistic to receive a reward in the MID and NP chambers than fish from LD tanks. We hypothesized that access to more space was preferable over large group sizes, yet our results indicate the contrary. Little is known about the preferred group sizes of rainbow trout in a semi-natural setting[390,391]. In the wild, however, low population densities often result in territorial defense and dominant fish driving out subordinate fish from a preferred area, while higher population densities lead to the formation of fish aggregates[81]. Therefore, it is possible that fewer territorial interactions occurred in the high-density treatment, improving their affective state. Our findings suggest that housing rainbow trout in small groups at high densities results in fish that are optimistic in novel situations, therefore in a more positive affective state than fish housed in small groups at low densities.

One limitation of our study was the time-intensive judgement bias training process. In order to be practically useful, judgment bias measures need to be easily attainable. We recommend further study into the modification of the judgement bias cues to be more biologically relevant. Perhaps utilizing access to conspecifics and social isolation as reward and punishment cues may allow for quicker training and a larger sample size for testing, however this has not yet been investigated.

Previous studies have found positive effects of tank complexity on performance parameters of rainbow trout[191,192,194,195]. In the present study, neither environmental complexity nor stocking density impacted body weight or length of fish. The lack of effect of the former could be due to the length of exposure to these enrichments. Vertically-suspended aluminum angles or rods with varying enrichment exposure duration (51, 61, 110, or 141 days) were associated with better trout weights, lengths, tank weight gain, and feed conversion ratio compared to trout housed in barren tanks[192-

[195]. Similarly, tanks with hanging colored plastic balls (for 70 or 127 days) showed improved tank weight gain, feed conversion ratio, and individual weight and lengths compared to trout housed without enrichments[191,392]. The previous work shows longer exposure times than the current study, which could be the reason for the lack of impact of complexity on production parameters in the current study.

The lack of effect of stocking density on production outcomes is somewhat in line with earlier findings[393,394]. For instance, rainbow trout reared at either 10, 40, or 80kg/m^3 did not differ in growth rate[107]. Contrary to our findings, 70% of reviewed publications reported adverse effects of high densities (similar or higher than the density in the current study) on trout growth (see review[87]). For example, rainbow trout housed at 312 fish/m^3 had the worst growth rate compared to trout housed at low densities of either 31, 94, 156, or 250 fish/m^3 (compared to 165 fish/m^3 versus 69 fish/m^3 in the present study), with no differences in growth rate between the lower density levels[395]. Based on the judgement bias test responses in HD tanks and the lack of effects of stocking density on production outcomes, we can conclude that the high stocking density in this study was not detrimental to trout welfare or production up to day 96.

Feeding activity has been used widely as an observational indicator of fish welfare, as stressors can reduce feed intake and motivation to feed[119]. For instance, too low stocking densities decreased feed intake in rainbow trout[87]. We predicted that fish from HD tanks would have an increased motivation to feed, as increasing group size has shown to increase food-seeking behavior[390]. Our results conform to our predictions, as fish from HD tanks began feeding faster than fish from LD tanks (0.25s compared to 0.79s). This suggests that fish from HD tanks either had an increased motivation to feed or that fish from HD tanks were less stressed compared to fish from LD tanks. Although plasma cortisol concentrations did not significantly differ between stocking density treatments, numeric values do show a similar response compared to feeding behavior latencies, with fish from HD tanks having lower plasma cortisol than fish from LD tanks. This could imply that the fish from HD tanks were less stressed (lower cortisol) and more motivated to feed (shorter latencies to begin feeding) than fish from LD tanks, suggesting improved welfare.

Previous work on impacts of stocking density on cortisol levels show varying outcomes within and between studies. We hypothesized that fish from HC/HD tanks would have the lowest levels of plasma cortisol, as environmental complexity has been shown to reduce the impact of environmental stressors, while too low of stocking densities can result in higher stress levels[190,396]. Opposite to our predictions and to some previous work, treatments did not impact plasma cortisol levels. Atlantic salmon reared with plastic tubes or shredded black plastic bag enrichments had lower basal plasma cortisol levels than salmon reared in a barren environment (approximately 35ng/mL compared to 10-15ng/mL)[382]. Rainbow trout reared at a low density of 134g/L (compared to 73.5g/L in the present study) showed higher plasma cortisol levels compared to fish reared at a high density of 277g/L (approximately 18ng/mL versus 5ng/mL)[396]. Similarly, rainbow trout reared at 10kg/m^3 showed higher plasma cortisol concentrations than trout reared at 80kg/m^3 during 5 of the 9 sample timepoints[107]. Unstressed (control) brown trout (*Salmo trutta*) exhibited variable plasma cortisol concentrations, from approximately 20-70ng/mL over the span of 8 hours[397]. In contrast, basal levels of plasma cortisol in unstressed salmonid fish have been reported to remain between 0-5ng/mL[398]. This variation may, in addition to species and strain differences, be caused by the inconsistent nature of cortisol responses[398–401], suggesting it may not be a reliable indicator for animal welfare.

This study is the first to establish the effect of environmental complexity and stocking density on judgement bias (optimism) in rainbow trout. Our results indicate that housing rainbow trout in relatively small groups at high densities from day 64 through 96 results in improved welfare status without any negative effects on performance parameters. The high stocking density level (165 fish/m^3) used in this study resulted in more optimistic responses during the judgement bias test compared to fish in low-density environments, therefore suggesting a positive affective state in the former. Further confirmation of the beneficial effect of high density was the increased motivation to feed compared to fish housed at low densities. Therefore, trout feeding behavior shows potential as a feasible animal welfare indicator in a production setting, as it can be easily measured by aquaculture personnel. Monitoring changes in feeding behavior could be a useful indicator of a health or welfare issue. By housing rainbow

trout in the density conditions described in this study and monitoring feeding behavior regularly, producers have the opportunity to rear fish under high welfare standards.

This study showed that a group approach to judgement bias training and testing resulted in differences between density treatments suggestive of a positive affective state for trout in high stocking densities. More research is needed on effective environmental enrichment and duration of exposure to enrichments for rainbow trout. With further investigation and modification of the test approach, judgement bias tests can be a valid indicator of affective state in rainbow trout.

5.5 Materials and Methods

Ethics

This experiment was approved by Virginia Tech's Institutional Animal Care and Use Committee (20-074) and was conducted in the Department of Food Science and Technology's aquaculture facility at Virginia Tech's Human, Agriculture, and Biosciences Building I from August to December 2020. This experiment was performed in accordance with the Institutional Animal Care and Use Committee's relevant guidelines and regulations.

Subjects and housing

Rainbow trout (n = 108; F1 generation Shasta strain) were bred and hatched in January 2020 and cultured at the Wytheville State Fish Hatchery (Wytheville, VA). In August 2020, fingerlings were transported to the research facility in 2 tanks of 14.1°C water. Upon arrival, fingerlings were acclimated to the recirculating aquaculture system water conditions (13.3°C) and facility management for 4 weeks. No mortalities were observed during or 24 hours post-transportation. On day 1 of the experiment, fish were distributed across 5 tanks (45.7 x 73.7 x 21.6 cm; water volume = 0.0726m^3) under commercial conditions with 21, 22, or 23 fish in each tank. Temperature, dissolved oxygen level, ammonia-N, nitrite-N, nitrate-N, pH, and alkalinity were monitored at

least once per week. All water quality parameters remained within suitable ranges[402,403], with the exception of alkalinity, which dropped below the optimal range on day 3. Sodium bicarbonate was added to the system throughout the trial to maintain optimal alkalinity values. Fish were fed a commercial trout diet (3 mm Finfish Gold, Zeigler Bros Inc., Gardner, PA, USA) once daily ad libitum. A subsample of fish (n = 72) were tagged with T-bar tags (Floy Tag, WA, USA) after sedation with sodium bicarbonate buffered MS-222 (Syndel, Ferndale, WA, USA) on days 1-3[404].

Treatments

This experiment involved a 2×2 factorial design using environmental complexity and stocking density as factors, resulting in 4 treatment groups: HC/HD, HC/LD, LC/HD, and LC/LD. All fish (n = 108) were kept under the same commercial conditions (5 tanks) until day 64 of the experiment. During this time, all fish were trained on a judgement bias task at tank level. After the judgement bias training was completed and on day 64, 40 fish from the 2 tanks that were successfully trained were allocated to 8 treatment tanks (5 successfully trained fish/treatment tank). Tags of successfully trained fish were marked with black marker (n = 40) to differentiate them from fish that were not successfully trained, most importantly in HD tanks. Three arbitrarily selected fish from the 2 successfully trained tanks were excluded from the experiment to achieve an even distribution of successfully trained fish across the 8 treatment tanks. Twenty-eight fish from the remaining 3 tanks that did not meet the learning criterion were arbitrarily selected and evenly distributed across 4 of the 8 tanks for the HD treatment (n = 7 randomly selected fish/HD tank). The remaining fish (n = 37) were excluded from the experiment. Thus, from days 1-64 all fish were kept under commercial conditions in 5 tanks, then a subsample of fish (n = 68) were redistributed over 8 treatment tanks in which they remained until day 96 (Figure 5.4).

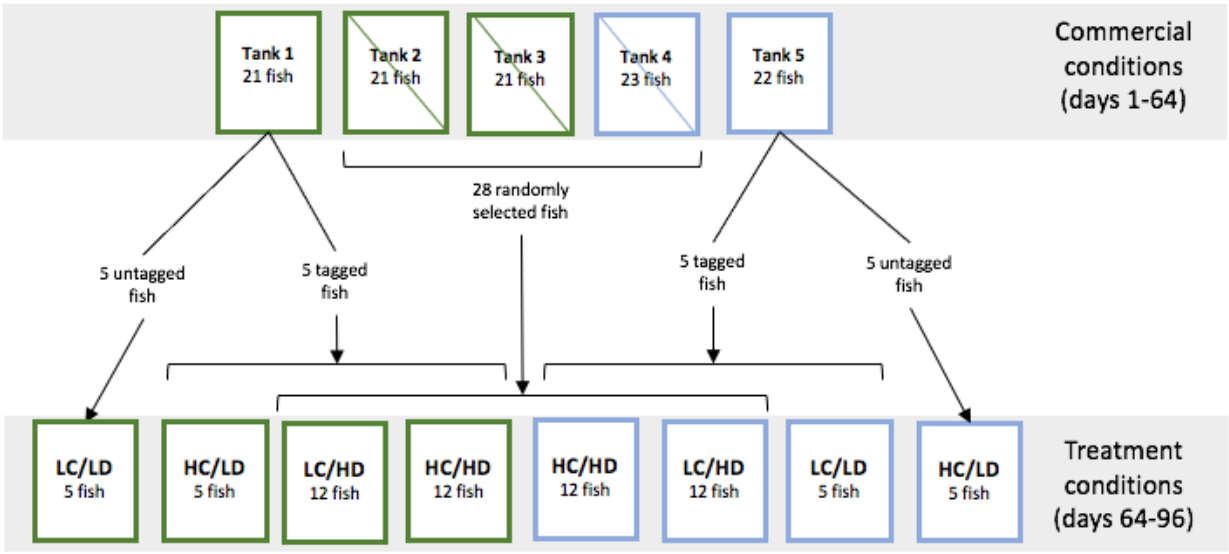

Figure 5.4. Overview of housing conditions and fish distribution throughout the experiment. Tanks in green were trained with the positive cue on the far left of the judgement bias arena, while tanks in blue were trained with the positive cue on the far right. Tanks with fish that did not pass phase 3 learning criterion are crossed with a diagonal line. LC = low-complexity, HC = high-complexity, LD = low-density, HD = high-density treatments.

Four tanks provided a complex environment (HC), while the other four tanks provided a simple environment similar to commercial standards (LC). HC tanks contained one PVC shelter structure (cut in half, 10.2cm diameter, 15.2cm long), which was placed at the bottom of the tank, two artificial floating lily pads (17.5cm x 17.0cm, Amazon.com, Inc., WA, USA), and two artificial cabomba plants (17.8cm, AquaTop, CA, USA). Enrichment objects were removed and disinfected daily. LC tanks contained no enrichment objects.

Based on the mean fish weight of 287g at day 96 and the Food and Agriculture Organization of the United Nations guidelines[373], 4 tanks (HC = 2; LC = 2) were stocked at a high-density (HD) of 12 fish (165 fish/m^3). The other 4 tanks (HC = 2; LC = 2) were stocked at a low-density (LD) of 5 fish (69 fish/m^3).

Judgement bias test

The judgement bias process followed a 7-step approach (habituation; training: phase 1, phase 1A, phase 2, phase 3; reminder training; and testing; Table 5.2) and took place in the home tanks, at tank-level. All steps of the judgement bias test were performed using a removable plexiglass arena with 5 equally-distanced opaque blue chambers and sliding doors (Figure 5.5). Sliding doors allowed for the experimenter to provide or deny access to each of the 5 chambers in the arena.

Table 5.2. Overview of judgement bias training and testing approach for rainbow trout during days 13-95. Fish (n = 5 tanks; 108 fish) were trained to associate a positive reward (handful of feed) with the POS chamber and a negative punishment (1s chase by net) with the NEG chamber. A subsample of fish (n = 2 tanks; 38 fish) moved on to the reminder training and testing phases.

Judgement bias phase	Objective	Day[1]	Maximum duration/ session (min)		Attempts/ session (n)	Measurements
Habituation	Acclimate fish to arena in groups of ~21	13-31	Sessions 1-4	5	1	n/a
			Sessions 5-9	15	1	
1	Associate POS chamber with reward in groups of ~21	32-58	5		12	Fish that entered chamber (n), fish feeding (y/n)
1A	Autoshaping in groups of ~21	39-57	5		12	Fish that oriented towards or entered chamber (n), fish feeding (n)
2	Enter POS chamber while in groups of ~10	49-59	5		12	Fish that entered the chamber (n), fish feeding (n), latency of first fish to enter (s)
3	Associate NEG chamber with punishment	59-61	2.5		6	Latency for the first fish to enter (s)
Reminder training[2]	Reinforce NEG and POS chamber					Latency for the first fish to enter (s)

	responses	71, 79, 88	2.5	6	
Testing[2]	Record responses to ambiguous cues	92, 93, 95	4	7	Latency for the first fish to enter (s)

[1]From start of the phase until the last tank met the learning criterion for that phase

[2]Occurred after fish were allocated to their treatment groups

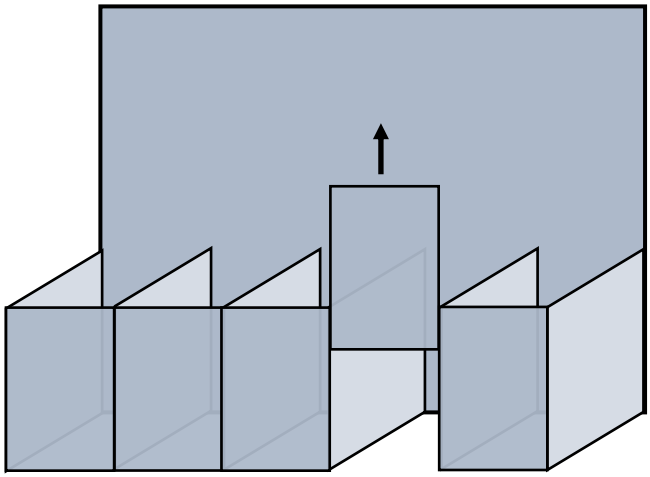

Figure 5.5. Diagram of the judgement bias arena. Positive (POS) and negative (NEG) chambers were balanced across tanks (n = 8). The middle three ambiguous chambers were introduced during testing. Entrance into each chamber was allowed by opening a sliding plexiglass door.

Habituation to the judgement bias arena without sliding doors was performed with all fish within a tank (n = 21-23 fish/tank), including the subsample of tagged fish (Table 5.2). A single experimenter gently placed the arena in the tanks from days 13-31 for 9 habituation sessions. For the first 4 sessions, the arena was placed into each tank for 5min with the experimenter out of sight. For the following 3 sessions, the arena was placed and the experimenter remained in line of sight for 15min per session. During the last 2 sessions, the arena was placed and the experimenter sprinkled feed into the tank, remaining in line of sight for 15min per session.

Training phases 1-3, reminder training, and testing involved placing the arena into each tank with the sliding doors closed for 3min to allow fish to acclimate. If a fish entered the POS chamber within any 15s attempt during phases 1-3, feed was immediately placed into the chamber and fish were allowed 10s to feed. After every attempt, the chamber door was closed and remaining feed was removed. Habituation and phases 1-3 were performed while fish were housed in commercial conditions (n = 21-23 fish/tank), while reminder training and testing took place while fish were housed under treatment conditions (Table 5.2).

Phase 1 of training was performed at tank-level (Table 5.2). Fish in each tank (n = 21-23 fish/tank) were trained to associate a chamber on either the far left (3 tanks) or far right (2 tanks) side of the arena with a reward (POS; approximately 30 feed pellets). If no fish entered the chamber within 15s, feed was placed into the chamber and 10s was allowed for fish to enter and feed. The learning criterion for phase 1 was met when at least 1 fish entered the POS chamber within 15s for 9 out of 12 attempts during two consecutive phase 1 training sessions. Two tanks passed the learning criterion for phase 1 of training between days 38 and 58, the other three tanks moved on to phase 1A of training, as the fish were not close to meeting the learning criterion after 5 sessions. Training phase 1A was similar to phase 1, however, fish were rewarded with feed if they oriented towards or swam within 15cm of the POS chamber opening. Tanks returned to phase 1 of training once at least 1 fish entered the POS chamber within 15s during two consecutive phase 1A training sessions. Only one tank met the learning criterion for phase 1A and returned to phase 1 on day 46, meeting the phase 1 learning criterion on day 47. The other two tanks remained in phase 1A until day 57, when they were excluded from the judgement bias task due to unresponsiveness.

Phase 2 of training with the remaining three tanks was aimed to habituate fish to being in a smaller group within their tank (approximately 10 fish/group; 2 groups/tank; Table 5.2). Half of the fish (group 1) were gently herded to the side of the tank containing the arena and separated from their conspecifics (group 2) by placing a blue opaque plexiglass separator into the middle of the tank. Then, the POS chamber door was opened. If no fish entered the chamber within the first 15s attempt, feed was placed into

the chamber and fish were allowed 10s to enter and feed. If no fish entered during any of the following 15s attempts, the chamber door was immediately closed and the next attempt began. If a fish entered the chamber within any of the 15s attempts, feed was immediately placed into the chamber and fish were allowed 10s to feed. After group 1, group 2 underwent the same training session. Phase 2 learning criterion was met when at least one fish in both groups 1 and 2 entered the POS chamber within 15s and consumed feed in 9 out of 12 attempts during two consecutive phase 2 sessions. All three tanks passed the learning criterion for phase 2 of training between days 45 and 59.

In training phase 3, the negative chamber was introduced (NEG; net placed in water for 1s; Table 5.2). NEG and POS cue presentations were pseudorandomized according to a predetermined order, never allowing more than two consecutive presentations of the NEG or POS cue, and began and ended with the POS cue. Half of the fish (group 1; approximately 10 fish) were gently herded to the side of the tank containing the arena and separated from their conspecifics (group 2; approximately 10 fish) by placing the blue opaque plexiglass separator into the middle of the tank. Then, the POS chamber door was opened. If a fish did not enter within 15s, the door was immediately closed. When the NEG chamber was opened, fish were allotted 15s to enter the chamber. If a fish entered within 15s, a green net was placed into the water for approximately 1s, then the chamber door was closed. If no fish entered within 15s, the chamber door was immediately closed. After 6 attempts (1 session) for group 1, group 2 was trained. Phase 3 learning criterion was met when at least one fish from both groups enter the POS chamber 100% of the time it was accessible and neglected to enter the NEG chamber 100% of the time it was accessible during two consecutive phase 3 training sessions. Two tanks passed the learning criterion for phase 3 on day 61. The third tank was excluded from the judgement bias task due to time constraints.

The trained subsample of fish (HD) or all fish (LD) underwent weekly reminder training sessions identical to phase 3 of training (Table 5.2). In HC tanks, enrichment objects were removed prior to a session.

Each of the 8 tanks (n = 38 fish) were tested for judgement bias three times on days 92, 93, and 95 (Table 5.2). In addition to the POS and NEG cues, three ambiguous cues (near

positive, NP; middle, MID; near negative, NN) were individually presented at intermediate locations within the arena (near left, middle, and near right; Figure 5.5). Each ambiguous cue was presented once per session according to a pre-determined order. Fish were given 20s to enter an opened chamber. Testing always began and ended with the POS cue, which was presented 8 times with fish receiving a feed reward if they entered the chamber. The NEG cue was presented 4 times and if a fish entered the chamber, it was chased by a net for 1s. All ambiguous cues were neither rewarded nor punished. For HC tanks, enrichment objects were removed prior to testing. In HD tanks, the tagged subsample of fish was separated from the other 7 fish with a blue plexiglass separator throughout testing. Fish were allowed 3min to acclimate. Latency for the first fish to enter each chamber (s) was recorded. A maximum latency score of 20s was appointed to attempts during which no fish entered the chamber.

Body weight and standard length

On days 64 (n = 68) and 96 (n = 66), individual weights and lengths were recorded. On day 96, only 66 fish were weighed and measured due to 2 mortalities. Fish were individually netted and placed into a buffered MS-222 water bath (sedation strength of 75-100mg/L) for approximately 5min or until sedated[404]. After sedation, the fish was placed on a scale (PTS3000, 0.1g precision, PESOLA, CH, USA) and then beside a ruler to measure standard length (from the tip of the nose to the base of the caudal fin). Then, the fish was immediately placed in a fresh water recovery tank where it was monitored until normal swimming behavior resumed. At this point, the fish was placed back into the home tank.

Feeding behavior

Latency to begin feeding was assessed at tank level on days 79-95 (observation n = 128). During daily feeding, the observer sprinkled a handful of feed into the tank and immediately started a timer. Latency until the first fish began feeding (s) was recorded for each tank once daily.

Plasma cortisol

On day 96, blood was collected from the 38 fish trained on the judgement bias task (n = 4-5/tank). Fish (n = 38) were individually netted and placed into a buffered MS-222 water bath (sedation strength of 75-100mg/L) for approximately 5min or until sedated[404]. After sedation, the fish was placed on a wetted work table and bled from the caudal tail vessel using a 23-gauge needle and syringe. Approximately 0.5-1.0mL of blood was obtained, then the fish was immediately euthanized by an overdose of buffered MS-222 (euthanasia strength of 250mg/L). Blood was placed in heparinized tubes and kept on ice until centrifugation. Samples were centrifuged at 3000 x g for 10min at 12°C, then plasma was stored at -80°C until ELISA analysis using a commercial cortisol express ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The ELISA was performed following the manufacturer protocol. Four of 38 samples were excluded from the statistical analysis due to unreliable assay results.

Statistical analysis

All data were analyzed in JMP pro 15 (SAS Institute Inc., Cary, NC, USA). Judgement bias data residuals were deemed normally distributed based on visual examination of normal quantile plots. Mixed models were used with complexity, stocking density, and test session as fixed factors, tank as a random factor, and latency to enter each chamber as the response variable. The interaction between complexity and stocking density was included as a fixed effect and removed if not significant. There was an effect of stocking density on latencies, so we blocked the analysis by chamber type (POS, NP, MID, NN, NEG) to assess the effect of density on latencies for each separate chamber. Tukey HSD post-hoc comparisons were used to evaluate pairwise differences. Length, weight, and plasma cortisol data residuals were normally distributed and analyzed using mixed models, with environmental complexity and stocking density as fixed factors and tank as a random factor. Latency to begin feeding data were log10 transformed to obtain a normal distribution of data residuals and are presented as raw means. Then, mixed models were used with environmental complexity and stocking density as fixed factors and tank as a random factor. Data are presented as least squares means ± standard error unless otherwise noted.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions statement

M.G.A. and L.J. designed and conducted the experiment together with S.A.S. and D.D.K. M.G.A., L.J. and A.M.C. performed statistical analysis on the data. M.G.A. and L.J. prepared the manuscript with assistance from S.A.S. and D.D.K. All authors have read and approved of the final manuscript.

Competing interests

The authors declare no competing interests.

Chapter 6. Discussion and conclusions

The research objectives were to determine whether environmental complexity and stocking density impacted: 1) broiler chicken affective state through a judgement bias test, 2) broiler chicken anxiety and fearfulness through attention bias and tonic immobility tests, and 3) rainbow trout affective state through a judgement bias test. To our knowledge, this research provides the first evidence of the beneficial effect of environmental complexity on broiler chicken affective state and validation of judgement and attention bias tests in broilers (Chapters 3 and 4). Providing broilers with a complex environment increased optimism and reduced anxiety, therefore positively impacted their affective state. This complex environment can stimulate positive, meaningful activities in broilers and reduce the occurrence of welfare concerns associated with genetic selection and a barren environment (i.e. contact dermatitis and poor leg health). Furthermore, a complex environment contributed to reduced anxiety, which can positively impact broiler health, as broilers may develop the ability to appropriately cope with perceived environmental threats (i.e. loud noises around the facility, transportation, or handling), minimizing the risk of a panicked response leading to injury, pain, and carcass bruising/scratching. It is generally accepted that fear in broilers can be minimized by providing enrichments, but we did not discover any impact of environmental complexity on fearfulness. Although we attempted to maintain novelty within the complex environment, the lack of effect on fear agrees with some previous reports. Alternatively, we found no effect of stocking density on broiler affective state, however, 3-week old broilers housed at high densities showed decreased fearfulness compared to broilers of the same age housed at low densities. Through further confirmation, housing young broilers in small areas of the house to keep stocking densities high and gradually increasing space allowances as birds age may have a beneficial effect on fearfulness and welfare. By giving broilers the choice to perform certain highly-motivated behaviors in preferred areas of their environment, such as perching on low, wide platforms or dustbathing in sand substrate, commercial operations can raise broilers in a positive affective state and under higher welfare status.

The novel judgement bias test approach used in Chapter 5 yields unexplored results regarding how environmental conditions impact rainbow trout affective states and welfare. Housing rainbow trout at slightly higher densities (165 fish/m^3) in small tanks proved to have a beneficial effect on their affective state, observed through optimistic responses during the judgement bias test. Rearing trout in this manner may not be feasible from an economic standpoint, however, Chapter 5 contributes much needed information on the effects of stocking density on rainbow trout welfare, which was previously unknown. Rainbow trout housed in low-density conditions responded less optimistically during the judgement bias test and exhibited a poor feeding response compared to trout housed in high-density conditions. In conjunction, these results establish that housing rainbow trout under low densities (69 fish/m^3) contributes to a negative affective state and poor welfare. The complex environment did not impact trout affective states, possibly because the enrichments were not provided for a long enough period or because stocking density overruled any potential beneficial effects of environmental complexity. A summary of the main findings from this thesis are summarized in Table 6.1.

Table 6.1. Summary of the main findings from Chapters 3, 4, and 5. 0 = no effect, - = reduced, and + = improved.

Species	Factor	Affective state measure	Treatment	Result
Broiler chicken	Environmental complexity	Fear	All	0
		Anxiety	High-complexity	-
		Optimism	High-complexity	+
	Stocking density	Fear	High-density	-
		Anxiety	All	0
		Optimism	All	0
Rainbow trout	Environmental complexity	Optimism	All	0
	Stocking density	Optimism	High-density	+

6.1 Recommendations for future research

Based on our findings from the presented studies, a number of recommendations for future research are listed below.

- Investigate biologically-relevant judgement bias cues for broiler chickens and rainbow trout

The novel judgement bias tests used in Chapters 3 and 5 provide a starting point for future research. These studies provide insightful results and validated the use of judgement bias tests in broiler chickens and rainbow trout, as both species were able to successfully discriminate between the two trained cues. However, training phases in both studies were time consuming, required ongoing modification, and were sensitive to variation. Future studies should consider biologically-relevant cues to potentially accelerate the judgement bias training phases and increase the sample size for testing. For example in rainbow trout, a positive cue that allows access to conspecifics and a negative cue that exposes fish to a bright light.

- Evaluate the effectiveness of a sedentary judgement bias task for broiler chickens

During judgement bias training and testing in Chapter 3, broiler chickens were required to walk approximately 1m to approach cues. Broiler walking ability was generally good in Chapter 3, observed through low gait scores, suggesting leg health did not impair birds' ability to approach cues during judgement bias training and testing. However, it is possible that the birds' heavy body weight and large size could have some painful effects on walking ability, even in birds with low gait scores. Furthermore, future studies may have a flock with higher gait scores compared to our study, and therefore encounter walking ability as a confounding factor while using the described judgement bias method. Because of this issue, evaluating the effectiveness of a sedentary judgement bias task may mitigate the potential negative impact of walking ability on broilers' ability to perform during judgement bias training and testing. One previous method used in laying hens which could be feasible to implement with broilers is a screen-peck task[241]. In this method, a digital computer screen is used to present visual cues (e.g. dots

of different colors) within close proximity to the subject, eliminating the need for ambulation.

- Perform attention bias testing in older broiler chickens

In Chapter 4, we discovered no effect of stocking density on attention bias in broiler chickens, which was against our predictions. When stocking densities are higher than 34-38kg/m^2, broiler welfare may be compromised[47]. Because attention bias testing was performed when high densities ranged between 19-30kg/m^2, the potential detrimental effect of high stocking density on attention bias could have been absent. We recommend future studies perform attention bias testing later in life, when densities are at least 34kg/m^2 to determine if, in fact, high stocking density does have an effect on attention bias in broilers.

- Evaluate whether providing broilers with a complex environment improves their behavioral repertoire

The complex environment in Chapters 3, 4, and 5 provided animals with the opportunity to engage in highly-motivated, functional behaviors. Due to the increased potential for experiencing positive emotions (affective state), minimizing negative emotions, and living an environment more similar to their natural environment than commercial conditions (natural living), we hypothesized that animals raised in a complex environment would be in a positive affective state and show improved welfare compared to those raised in a barren environment. Details on the behavior of rainbow trout under experimental conditions are lacking, so future studies might consider the use of underwater cameras to determine if rainbow trout behavior is altered in a complex compared to a barren environment, and how so. While we did find a beneficial effect of environmental complexity on affective states of broiler chickens, we do not yet know the details of their interactions with the enrichment objects. Through behavioral observation of broilers in a complex environment and preference tests, future studies can determine if their behavioral repertoire is improved/expanded, which enrichments are preferred for performing specific behaviors, and if broilers value a certain enrichment over others.

- Determine whether there is an age effect on fearfulness in broiler chickens

Our results from Chapter 4 indicated that broilers from high-density environments were less fearful than birds from low-density environments at 3 weeks of age, observed through shorter durations of tonic immobility. This was against our predictions, as previous studies indicate that housing broilers at high densities contributes to increased fearfulness (see references within page 69). No differences in fearfulness were found between high and low stocking densities used in a previous study which were comparable to those in our study, where we did find an effect of density on fear. However, the previous study performed tonic immobility testing at 6 weeks of age compared to 3 weeks of age in our study, which could be explained by an age effect on fearfulness in broilers. When broilers are young and small, they may place greater importance on high stocking densities that provides perceived natural protection through safety in numbers compared to older, larger birds. Therefore, we recommend future research to investigate the relationship between age and fearfulness in broilers.

- Evaluate the impact of environmental complexity on other broiler chicken welfare indicators

Our results from Chapters 3 and 4 indicate that providing broilers with a complex environment improves affective state and reduces anxiety, ultimately improving their welfare. While we can assume the subsequent effects of a positive affective state and reduced anxiety improve aspects of broiler health and biological functioning, a limitation of our studies is that the exact effects of a complex environment on these aspects of broiler welfare are unknown. In reference to Figure 2.1, we recommend investigation of a complex environment on outputs such as: lameness, disturbances of resting birds, contact dermatitis, and carcass quality.

- Determine the minimum duration of enrichment exposure to elicit a positive impact on affective states of rainbow trout

Results from the judgement bias test in Chapter 5 suggest there was no impact of environmental complexity on optimism or pessimism of rainbow trout. The effect of

enrichments on rainbow trout affective state has not been previously investigated, but affective states of other fish species has. Three previous studies report differences in judgement bias of fish in varying housing conditions, suggesting that judgement bias can be influenced by environmental condition. However, it is possible that trout in our study were not exposed to the enrichments for long enough to observe any effect on judgement bias, with 3 weeks of exposure prior to testing in our study compared to 7-18 weeks of exposure in previous work which found differences in production parameters. Future studies might consider determining the minimum duration of exposure to a complex environment (longer than 3 weeks) that would impact affective states of rainbow trout.

Evidence for the impact of environmental complexity and stocking density on affective states of broiler chickens and rainbow trout was previously uninvestigated. There is a beneficial effect of the described complex environment on affective states of broiler chickens, but not rainbow trout in the studied conditions. Stocking density had no impact on broiler chicken affective state, while there was a favorable effect of high stocking density on rainbow trout affective state, observed through optimistic responses in the judgement bias test. Commercial housing systems will continually improve with further investigation and validation of cognitive bias tests to evaluate affective states of these species. By raising the standard and altering our outlook on welfare by viewing an environment from the animal's perspective, we can provide broiler chickens and rainbow trout with an environment that ensures positive affective states and a life worth living.

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