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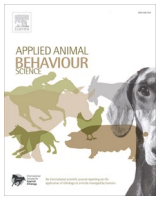

Training for a cognitive judgement bias task does not affect fear or telomere shortening in laying hens

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ABSTRACT

For behavioural tests such as the cognitive judgement bias task (JBT), animals usually undergo weeks of training involving various elements, such as handling, social isolation and learning the task. These may influence their affective state and other welfare indicators. Here, we investigated the effects of training for a JBT on fear-related behaviour in laying hens and a potential welfare indicator – telomere shortening. Hens were trained for a JBT (N = 16, across 2 batches), or kept as controls (N = 16, across 2 batches) that received no training or training-related handling. Hens that reached the learning criterion (N = 14) were tested in six JBT test sessions. Before and after the trained hens received JBT training, blood was collected from all hens, and they were assessed for fear-related behaviour by being subjected to open field, human approach, and tonic immobility tests. Relative telomere lengths (RTL; i.e., ratio of telomeric repeats versus a single copy control) were obtained from blood samples with a quantitative real-time polymerase chain reaction (PCR) method. As anticipation of the JBT sessions could affect the JBT outcome, we also recorded behavioural indicators of anticipation prior to each JBT test session. Except for an increased latency to first step in the open field test after training in the trained hens (t = 3.1, df=28, P = 0.012; *conditional* R^2=0.59), no other fear-related behaviour significantly differed in either group (P > 0.05) and RTL was not affected. In addition, none of the anticipation indicators predicted the JBT test outcome. Contra to our hypotheses, welfare-related aspects may not be strongly affected by the JBT training. However, the enriched housing of the hens from both treatments might have minimised the effect of training on fear and telomere shortening, or training may have affected indicators other than the ones we investigated. We conclude that because JBT training and testing does not affect fear assessment and telomere shortening outcomes in hens housed in enriched housing conditions, the same individuals can be used in welfare assessments involving similar assessments. Our results also suggest that the JBT itself may be robust against the temporary affective states associated with anticipation.

1. Introduction

Understanding and assessing affective states is a central concern of animal welfare science, but the subjective component of affective states is difficult to assess (Boissy and Lee, 2014; Mendl and Paul, 2004). Behavioural tests can be used to answer questions related to animals' affective states by assessing the behaviour component (Lagisz et al., 2020). For example, the cognitive judgement bias task (JBT) is used to assess the influence of an animal's affective state on appraisal and decision-making regarding ambiguous stimuli (Mendl and Paul, 2004; Mendl et al., 2010; Roelofs et al., 2016). However, days, or often weeks of training (depending on the task), are usually required to prepare animals for JBT testing which involve various elements, such as handling, social isolation, and learning the task. These elements may affect the welfare of the animal, including its affective states and other welfare indicators.

The extensive training required for a JBT may itself affect animal welfare (Browning, 2022; Roelofs et al., 2016). On the one hand, some of the training elements may induce negative affective states, such as fear (Forkman et al., 2007; Jones and Boissy, 2011). Fear is considered to be unpleasant and may lead to poor welfare if experienced over a long period of time (Duncan, 2004). Yet, only animals that successfully

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habituate to these elements (e.g., social isolation, handling) pass the training criteria (Roelofs et al., 2016). On the other hand, training for behavioural tests such as a JBT may provide cognitive enrichment. Cognitive enrichment can positively impact welfare and reduce fear-related behaviour as seen in goats (e.g., Oesterwind et al., 2016) and pigs (e.g., Puppe et al., 2007) when provided in the form of cognitive devices. As the same animals are sometimes used in both a JBT and fear assessments, (e.g., Stephenson and Haskell, 2022), results from fear assessments may also be affected if animals undergo the training for a JBT first.

Telomere shortening, a cellular biomarker of biological age, is a potential animal welfare indicator (Bateson and Poirier, 2019). Telomeres are repetitive sequences of DNA at the end of chromosomes that shorten with each cell division (Allsopp et al., 1995). In humans, negative life experiences result in shorter telomeres (Mathur et al., 2016; Pepper et al., 2018) and stress-related telomere shortening may decelerate by positive experiences (Schutte et al., 2020). Thus, telomere shortening can be used as a biomarker of welfare in humans. Importantly, telomeres shorten faster in people with higher perceived stress (Mathur et al., 2016), showing the significance of affective states on telomere length. Similarly, in non-human animals (including domestic chickens, Beloor et al., 2010; Sohn and Subramani, 2014) telomeres shorten faster due to negative experience (Chatelain et al., 2020), but information on the effects of the positive experience on telomere shortening is lacking.

Fear assessments and JBT are commonly conducted on domestic chickens (*Gallus gallus domesticus)*, which are the most abundant farm animal species on the planet. Nevertheless, no information could be found whether or to what extent training for a JBT affects fear-related behaviour or other welfare indicators (e.g., telomere shortening). Chickens are motivated to engage with cognitive enrichment (e.g., Schmelz and Krause, 2021). Thus, if JBT training is perceived as enriching, it is possible that training for a JBT could affect fear and other welfare indicators in chickens. Environmental enrichment, which introduces novelty and allows animals to engage with and exert control over their surroundings, and thus shares characteristics with JBT training, reduces fear-related behaviour in domestic chickens. For example, compared to chickens kept in a barren environment, young and adult enriched chickens express reduced fear-related behaviour when separated from the peers and placed in a novel environment (Jones and Carmichael, 1997; Suarez and Gallup, 1983) and shorter tonic immobility, another indicator of reduced fear (Jones, 1988, 1987). Moreover, regular human contact (a common element of JBT training) reduces avoidance and increases voluntary approach of young and adult chickens towards unknown humans, suggesting reduced fear of humans (Barnett et al., 1994; Graml et al., 2008; Jones and Waddington, 1992). Stress resilience also increased in adult chickens exposed to environmental enrichment (Ross et al., 2020), which could decelerate telomere shortening (Chatelain et al., 2020) and outcomes of fear assessments (Brockhurst et al., 2015). These findings suggest that training for a JBT could affect fear-related behaviour and telomere shortening in domestic chickens.

Based on chickens' experiences during JBT training, participating in regular training and testing sessions is likely to induce some level of anticipation (Anderson et al., 2020; Wichman et al., 2012), i.e., the expectation of future events based on previous experience. Anticipation of JBT sessions may alter the short-term emotions prior to training and testing (Mellor, 2015). Depending on how sensitive the JBT is with respect to the emotions associated with anticipation, the JBT outcome may be biased, though this has not yet been studied.

The objectives of this study were to investigate the effects of training and testing in a JBT on (1) fear-related behaviour and (2) telomere shortening. We also aimed (3) to investigate whether the level of anticipation shown prior to JBT testing sessions would affect decision-making under ambiguity (i.e., the outcome of the JBT), in adult domestic chickens (i.e., laying hens). We hypothesised that training and testing for a JBT would reduce fear-related behaviour, with no significant change in fear-related behaviours expected in control hens assessed in equal time intervals as the trained hens. Slower telomere shortening was expected in hens undergoing JBT training and testing compared to control hens. We also hypothesised that the intensity of anticipation indicators shown prior to the JBT test sessions would influence the JBT outcome. As we were unable to separate indicators of positive and negative anticipation, no prediction was made regarding the direction of the JBT outcome (i.e., whether hens showing stronger anticipation would be more optimistic- or pessimistic-like).

2. Animals, materials and methods

The study was approved by the Austrian Ministry for Education, Science and Research (GZ2021–0.116.834). All study procedures and manuscript development were performed with regard to PREPARE and ARRIVE guidelines (Du Sert et al., 2020; Smith et al., 2018). Data was collected at the University of Veterinary Medicine, Vienna, Austria, as part of a master thesis project (Neuhauser, 2023).

2.1. Animals and husbandry

Thirty-two Lohmann laying hens were used for the study in two equally sized batches and were 30 weeks (batch 1) or 24 weeks (batch 2) of age at the start of the study. Hens were marked for identification purposes using coloured leg bands and non-toxic livestock spray ("Raidex Animal Marking Spray", red, green, blue). All hens from a batch were housed together in a single home pen (3.2 m × 3.6 m, Fig. S1 in supplementary materials), equipped with two round feeders, two bell drinkers, two perches (0.25 m/hen; custom-made), a nest box (0.78 m L × 0.56 m W × 0.45 m H, transparent plastic box filled with straw), and a separation pen (1.0 m L × 1.0 m W × 1.0 m H; to be used for feed restriction during training). Wood shavings (5 cm deep) were provided as litter and were replaced weekly. Hens were exclusively fed laying hen mash feed ("Garant Legekorn Gegrützt"), which together with water was available in the home pen ad libitum (outside of feed restriction described under section 'JBT training and testing'). Enrichment in form of fresh feed either hung or scattered in litter was provided daily and additional enrichment items such as cardboard boxes or paper bags were exchanged bi-weekly. Daily husbandry was performed by JN, with one additional person for weekend care. Artificial light was provided for 16 h daily. Due to a technical error batch 2 hens were exposed to a 24-hour light period for two weeks after hens arrived at the facility, including during the time of the first fear assessments. We learnt about the error on day 1 of habituation and halted experimental procedures for one week to allow hens to re-settle before the study resumed.

2.2. Experimental design

All hens underwent the first fear assessment described under 'Fear assessment' at the start of the study. After this fear assessment, hens were allocated to two treatments: trained hens (N = 16 in total, 8 hens per batch) and control hens (N = 16 in total, 8 hens per batch) who did not receive any JBT training, testing, or additional handling outside of normal daily husbandry treatments. Allocation to treatment was based on the following indicators: duration of tonic immobility; the total duration of time spent within a 1 m proximity of a human during the human approach test, and the number of steps taken during the open field test (described under 'Behavioural analysis'). Allocation was based on high- and low-ranking birds with regard to the outcome of each indicator separately, with final group allocation determined so that groups were balanced insofar as possible for the above-mentioned indicators, across the two treatments. After allocation, trained hens received training and testing for a JBT. As each hen finished training and testing, that hen as well as one control hen underwent the 2nd fear assessment. As all hens were housed together in a single pen, all hens (i.e., control

and trained) experienced some form of human contact. This consisted of roughly 20 pen visits daily for catching hens to feed restrict them and bring them to the training and testing arenas, and roughly 30 min of daily husbandry. Affective state of the trained and tested hens in the JBT was not experimentally manipulated (i.e., no additional treatments were introduced).

2.3. Fear assessment

2.3.1. Fear assessment arena

Hens underwent fear assessments individually in an arena used only for fear assessments, constructed from timber framing in a corridor-style of 2.5 m L × 1.0 m W × 2.0 m H (Fig. S2 in supplementary materials). The walls on the inside of the arena were covered with dark green tarpaulin. The top half of the walls of the arena was marked every 0.5 m with a grey duct tape to aid measuring proximity during the human approach tests. Furthermore, the floor of the arena was covered with approximately 5 cm wood shavings to improve walking abilities, which were taken from the home pen to buffer isolation-related stress during testing. A white perforated plastic placement box (0.45 m L × 0.34 m W × 0.57 m H) was situated in the front-left corner of the arena and was used to ensure standardized placement of hens at the start of assessment. All tests were recorded using two wall-mounted camcorders (Sony CX730, CY900E), located at each end of the arena.

2.3.2. Fear assessment protocol

We assessed fear using a battery of behavioural tests: open field(OF), human approach/avoidance (HAT), and tonic immobility (TI) tests. To ensure maximum novelty during the open field test (Nielsen, 2020), the order of tests conducted within the fear assessment (OF, HAT, TI) remained the same for all hens and both fear assessments. For the 1st fear assessment, hens were haphazardly selected from the home pen for testing, and all tested across two days. For the 2nd fear assessment, each trained hen was assessed after she finished her last JBT test session, on the same day as one haphazardly selected control hen. A particular hen-pair underwent each fear assessment test on the same day with the order of hens within a hen-pair haphazardly selected each day. All fear tests were performed by the same experimenter (JN) to reduce handling differences (Forkman et al., 2007). Hens were manually carried from the home pen to the fear assessment arena in a standardized manner (left side of the hen gently held against the experimenter's chest, with the hen's head nestled under the experimenters left arm) to control for any effect of pre-test handling (Forkman et al., 2007). At the end of each fear assessment test, hens were returned directly to the home pen in the same manner as being brought to the arena.

If during any of the fear tests hens showed signs of extreme distress (i.e., ten jumps or ten distress vocalisations performed within 20 s), the assessment was stopped due to ethical concerns. These exclusion criteria were based on findings that, in an open field test, an average chicken emits four (adult) or six to seven (young) vocalisations within 20 s (Bari et al., 2021; de Haas et al., 2014; Widowski et al., 2022). Furthermore, in open field tests adult chickens perform less than one jump per minute (Campler et al., 2009; Uitdehaag et al., 2009). In line with our exclusion criteria, six fear tests were stopped (1st OF test: two control hens; 2nd OF test: one control and two trained hens; 1st HAT: one control hen), with hens subsequently being excluded from the statistical analysis for all affected indicators, excluding the indicator "first step", which was recorded prior to the tests' termination.

2.3.2.1. Open field test. The open field test was performed on day 1 from 0900 to 1100 h. Hens were placed in the placement box to standardise the starting location. After a ten-second period, the lights were turned on. Five seconds later, the box was lifted and secured 1 m above the hens' head at which time the OF test began and lasted for 2 min. Behavioural indicators of fear recorded included the latency to first step, the total number of steps, exploration, and the number of jumps and distress vocalisations and are described in Table 1. All behaviour was coded post hoc from the video recordings made in the fear assessment arena.

2.3.2.2. Human approach/avoidance test. Human approach/avoidance tests were adapted from home pen tests as applied by Graml et al. (2008) for a test arena and conducted on the same day as the OF test from 1400 to 1600 h. Hens were placed in the placement box with the front part of the hen's body facing towards the experimenter. The person was sitting cross-legged on the ground on the opposite end of the placement box when the tests started. After a ten-second period, the lights were turned on and five seconds later the placement box was lifted and secured 1 m above the hen. Once the placement box was fully raised, the human approach test began during which the hen could voluntarily approach

Table 1
Ethogram for assessed indicators during both fear assessments.

Indicators	Description
Open field test (OF)	
Steps (no.)	Lifting and placing down one foot in front of the body, resulting in the body moving in a forward motion (i.e., not including lifting and placing the foot back down in the same location).
First step (s)	The latency to take the first step, measured from when the placement box is fully lifted.
Exploration (prop.*)	Pecking (rapid touching of the beak on the ground or arena features) or ground scratching (moving foot in a rapid backwards motion against the ground). Ground scratching is often intermittent during bouts of pecking and often followed by one or two steps after ground scratch. Exploration bouts begin when the beak or front of the foot first contact the ground, and end after three seconds have elapsed without pecking or ground scratching.
Jumps (no.)	Hen is in an upright position and flaps its wings rapidly until lifting off the ground.
Distress vocalisations (no.)	Short, high-pitch and -volume vocalisations, often occurring at the end of a bout of lower-pitch and -volume vocalisations, but also occurring as a single vocalisation.
Human approach/avoidance tests (HAT)	
Human approach test	
First step (s)	Same as above
Touch (Y/N)	Any part of the hen's body, including the beak, in physical contact with any part of the human.
Proximity (prop.*)	The whole hen's trunk within the two quadrants of the test arena, closest to the test human. Time started when the front foot and the whole hen's trunk crossed the quadrant line and ended when one foot and the majority of the body passed back over it.
Avoidance test	
Avoidance distance / ADT (m)	The distance at which the hen withdrew from an approaching unfamiliar human, defined as when both feet stepped aside or away in any direction, i.e., changes in the body axis, turning away of the head, and the lifting and staying on one foot were ignored. The distance was scored based on number of the quadrants between the person and the hen. If the person could touch the hen or if the hen and the front foot of the person were within the same quadrant, the ADT was recorded as 0.0 m. Otherwise, the ADT was recorded in 0.5 m increments, with a maximum distance of 2 m.
Tonic immobility (TI)	
Attempts (no.)	The number of attempts required to successfully induce tonic immobility (minimum 1, maximum 5). Successful inductions are defined as no righting within 10 s after hand removal or less than 5 s of attempts to righten themselves during induction.
Duration (s)	Latency for the hen to right herself after tonic immobility was successfully induced, starting immediately after hand removal. Righting defined as the body being upright with both feet in contact with the cradle and/or floor.

* proportion of 2-minute test period spent displaying exploration or in proximity of human.

(or not) the experimenter, which lasted for 2 min. After the human approach test, the experimenter slowly stood up and 5 s later, the avoidance test began. In the avoidance test the experimenter approached the hen, moving at a speed of one step per second, until the hen withdrew, with the avoidance distance (ADT) measured (Butterworth et al., 2009; Graml et al., 2008). Behavioural indicators of fear recorded included the latency to first step, whether the hen touched the experimenter or not, the proportion of time spent close to the experimenter, and the avoidance distance (Table 1).

An unfamiliar female person wearing dark green coveralls, standard blue shoe covers, and a black FFP2 face mask performed the tests. The person gazed at the timer in their lap during the human approach test and straight ahead during the avoidance test. As animals' approach and avoidance behaviours reflect their perception of that person (Graml et al., 2008; Waiblinger et al., 2006), the same experimenter was used for both fear assessment time points where possible, to ensure that hens' behaviour in the 2nd fear assessment was not affected by being tested with a different person. Due to illness a different test person was used for nine hen-pairs in the 2nd fear assessment, who participated for both hens within a hen-pair. All experimenters were trained beforehand to ensure standardisation of tests and were blinded to the treatment group of the hens.

2.3.2.3. Tonic immobility test. The tonic immobility test was performed from 0900 to 1100 h on the day after OF and HAT tests were completed and was adapted from Larsen et al. (2018). A V-shaped cradle constructed from plastic-coated cardboard (0.53 m L x 0.24 m W x 0.16 m H) sat directly on the floor at the entrance of the arena. A hen was inverted on her back in the cradle and restrained for 15 s with one hand covering the head and the other over the breast area of the hen. The experimenter removed her hands, stood up, and moved away from the hen to a distance of 1.0 m, outside of the arena, with the hen's feet towards the experimenter. The experimenter gazed at her feet until the hen righted itself. Induction was considered successful if the hen remained lying down for 10 s after hand removal. If the hen righted herself before this time, induction was re-attempted, with a maximum of five total attempts. If the induction was successful, a maximum time of 10 min was allowed after which the hen was gently righted. Behavioural indicators of fear recorded included the duration of tonic immobility and the number of attempts needed to successfully induce tonic immobility (Table 1).

2.4. Judgement bias task training and testing

2.4.1. Judgement bias task arena

The JBT arena was located in a separate room within the same building. The walls of the JBT arena (1.2 m L × 1.0 m W × 0.7–1.5 m H; Fig. 1, below, and Fig. S3 in supplementary materials) were made of wood (wall containing reward locations: 1.5 m H, other walls: 0.7 m H) and the rest of the walls were made of a white tarpaulin material, with one side that could be opened by the experimenter. Within the JBT arena, five reward locations (henceforth 'goal-holes') were located on one wall of the arena, spaced equidistant from each other. Each goal-hole (0.1 m × 0.1 m) could be opened and closed manually by the experimenter sliding up a door covering the goal-hole from outside the arena and served as a spatial cue. Additionally, the insides of the goal-holes were coloured red, which contrasted with the wood colour of the apparatus and could only be seen by a hen once a goal-hole was open. The outermost goal-holes were designated as the positive and negative reference while the three intermediate goal-holes were designated as ambiguous (Near Positive, Middle, Near Negative). At the opposite end of the arena to the Middle ambiguous goal-hole, a 25 mm

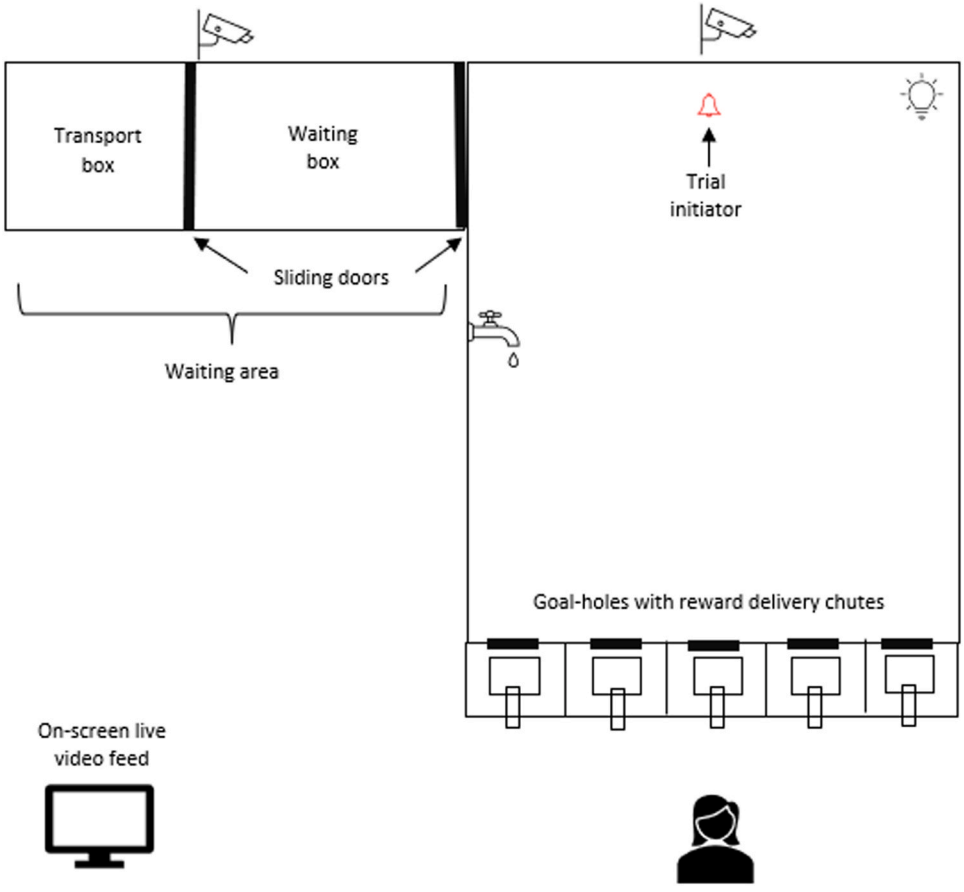

Fig. 1. Judgement bias task training arena. Removal of the left sliding door allowed hens to use the entire waiting area, and removal of the right sliding door allowed access into the JBT arena. Hen behaviour was video recorded via two ceiling-mounted cameras. Inside the JBT arena, hens had access to water (tap icon) and were trained under artificial light positioned in the back corner (light icon). A suspended red bell (bell icon) was used as a trial initiator and was operated by the experimenter standing outside the arena via a pulley system (person icon). Five equally spaced goal-holes were located opposite the trial initiator, each with its own sliding door, operated by the experimenter, as well as a PVC pipe allowing the experimenter to deliver a mealworm reward into a plastic cup inside an enclosed compartment. Experimenter observed hens via an on-screen live video feed.

red metal bell was suspended from the ceiling and operated as a trial initiator, which could be raised to approximately 1.0 m and lowered to approximately 0.3 m above the ground by the experimenter from outside the arena. Hens were trained using a reward stick (custom-made) which consisted of a 1.5 m wooden stick with a 10 × 10 cm see-through plastic cup attached to the end.

The hens were trained under artificial light positioned in the opposite end of the arena to the goal-holes, positioned in the right-hand corner of the JBT arena, with all other lights in the room turned off. Water was provided ad libitum inside the JBT arena via a small hanging cup and a playback recording of the home pen vocalisations was played for the entire session in the background to minimise social isolation-related stress. The same playback was used for all hens and for every training and testing session.

2.4.2. Waiting area

A wooden transport box (0.40 m L × 0.4 m W× 0.51 m H; Fig. 1) with a transparent lid covered with fabric during transport and one side designed as a sliding door was used to transport hens one-at-a-time to the arena. The transport box was placed on the ground with the sliding door against a waiting box (0.6 m L × 0.4 m W × 0.51 m H) next to the JBT arena. When the sliding door was opened it transformed the transport and waiting boxes into a larger space (henceforth 'waiting area'). At the far end of the waiting area from the transport box, another sliding door allowed access into the JBT arena.

2.4.3. Judgement bias task training and testing protocol

The training and testing protocol for the JBT was based on that developed by Hintze et al. (2018) and was adapted for laying hens in a pilot study. The protocol integrated active trial initiation into a spatial Go/No-go task, whereby physical initiation from the hen by pecking at the red bell (i.e., trial initiator) resulted in the opening of a goal-hole. Hens were trained to associate one reference goal-hole (left or right, depending on the hen) with a reward and to thus go there and the reference goal-hole on the other side (right or left) with no reward and thus not to go there or to re-initiate a new trial. The protocol consisted of four training stages: 1) habituation, 2) shaping for active trial initiation, 3) left-right discrimination, and 4) Go/No-go discrimination, followed by testing for judgement biases. To increase training motivation, hens were placed inside the separation pen with no access to food for two hours prior to starting JBT training and testing sessions (excluding habituation), but still had visual contact with other hens and ad libitum access to water. Hens were trained during weekdays by the same experimenter (JN) and received three training sessions per day during the habituation and one training session per day during the following stages. Training sessions lasted for a maximum of ten minutes during habituation and a maximum of 45 min during all following stages, including testing. Care was taken to ensure that hens were trained only when motivated. Thus, sessions were terminated after five minutes of non-performance (defined as no food consumption from the reward stick or goal-hole and no trial initiation) or after ten distress vocalisations or jumps within 20 s. One trained hen from batch 1 sustained a minor beak injury within the home pen. Training (stage 2; see below) for this hen was halted for one week to ensure healing and to prevent a negative association between training and pain.

2.4.3.1. Stage 1: Habituation. A covered metal separation pen (1.0 m L × 1.0 m W × 1.0 m H) used for feed restriction purposes was located inside the home pen. Hens were gradually exposed to being placed in the separation pen, the transport box, and being transported to and placed down next to the waiting box. After this, removal of the sliding door between the transport box and waiting box allowed hens to access the JBT waiting area where hens were habituated to spending 30 s before being allowed to enter and explore the JBT arena via another sliding door. Mealworms were scattered throughout the waiting area, the JBT arena, in the stationary reward stick and the open positive reference goal-hole to encourage exploration. The position of positive reference goal-holes was counterbalanced across hens, i.e., for half of the hens the farthest left reference goal-hole was positive and for the others the farthest right. Lastly, hens were incrementally exposed to the opening and closing of the positive reference goal-hole, moving of the reward stick, and raising and lowering of the trial initiator until staying calm, showing no stress-indicative behaviour, and eating mealworms from within the arena and positive reference goal-hole for two consecutive sessions. Hens moved from habituation to shaping once they were readily consuming mealworms from within the arena and positive reference goal-hole and showed no stress-related behaviour to raising and lowering of the trial initiator and moving of the reward stick. One hen from batch 1 was excluded from training as she did not show motivation to engage with the training protocol (i.e., failing to eat mealworms and consistently entering the arena).

2.4.3.2. Stage 2: Shaping for trial initiation and Go-response to the positive reference goal-hole. Shaping occurred gradually, starting with shaping to initiate trials. To encourage hens to peck at the initiator, mealworms were first attached to the trial initiator using tape. Each correct response was rewarded with mealworms from the trial initiator and additionally from the reward stick. When hens performed 20 self-initiations within one session, we stopped attaching mealworms to the trial initiator and a trial initiation was rewarded only with mealworms from the reward stick. Once hens were reliably pecking the initiator and consuming mealworms from the reward stick, the reward stick was moved gradually further away from the initiator in small increments towards the positive reference goal-hole, and finally with the reward placed directly inside the positive reference goal-hole container. Hens were moved to the next stage after correctly initiating at least 20 trials followed by Go responses and consumption of the rewards within a single session. During training and testing sessions, a Go response was recorded when the hen's head passed through the goal-hole, and a No-Go response was recorded if the head did not pass through the perimeter of the goal-hole, or if they re-initiated the trial.

2.4.3.3. Stage 3: Left-right discrimination. Hens were trained to show Go responses to both positive and negative reference goal-holes, with each session consisting of 50 self-initiated trials. Thus, both reference goal-holes were rewarded. This ensured that hens paid attention to any open goal-hole, rather than only differentiating if the positive reference goal-hole is opened or not (Hintze et al., 2018). After initiation, a positive or negative reference goal-hole was left open for a maximum of 15 s, after which it was closed. Hens moved to the next stage after performing 80% correct Go responses to both positive and negative reference goal-holes for two consecutive sessions. Hens were excluded from this stage onwards if they did not complete all 50 trials within a session for five consecutive sessions, with one hen from batch 2 being excluded.

2.4.3.4. Stage 4: Go/No-go discrimination. The positive reference goal-hole remained rewarded, whereas the negative reference goal-hole was no longer rewarded. Hens finished stage 4 and moved to testing for judgement biases after performing 80% correct responses to both positive (Go response) and negative (No-Go response) reference goal-holes across four consecutive 20-trial blocks. In batch 1 all hens approached the negative reference goal-hole more than once in at least one test session and four out of seven hens approached the negative reference goal-hole in at least two sessions; the average percentage of incorrect responses for the negative reference goal-hole across all test sessions and hens was 6.5%. Thus, for batch 2 hens during stage 4 we introduced a mild punishment in form of a 'time-out period' lasting eight seconds, during which the light in the arena was turned off for five seconds and the trial initiator remained unavailable for further three

seconds. This reduced incorrect responses in batch 2 with an average percentage of incorrect responses for the negative reference goal-hole across all test sessions of 1.77%.

2.4.3.5. JBT testing. Judgement biases were assessed for each trained hen across six test sessions (one per day), lasting for a maximum of 45 min. Each test session consisted of the opening of each reference-goal hole 25 times and opening of the three ambiguous goal-holes (Near Positive, Middle, Near Negative) once per session in trials 16, 32, and 48 in a counterbalanced order across the sessions. Additionally, the order of the reference goal-holes presented before ambiguous goal-holes was balanced so that ambiguous goal-holes were presented equally often after both the positive and negative reference goal-holes. Since animals showing a Go response in ambiguous cues expect to receive a reward, we decided to reward these responses (i.e. reward according to expectation) to avoid surprising reward omissions.(Hintze et al., 2018; Papini and Dudley, 1997). Go responses to the negative reference goal-hole remained either non-rewarded (batch 1) or non-rewarded with a time-out (batch 2).

During the first two stages of training, hens could see the experimenter from one side of the arena as the white tarpaulin sheet needed to be removed in order to present the reward stick or to remove hens from the arena at the end of sessions. From the end of stage 2 of the JBT training onwards (including JBT testing), hens had no visual contact with the experimenter and the experimenter could only observe the hens via a live video feed from two ceiling-mounted cameras (Sanyo VCC-HD2300P; GeoVision Surveillance Software V8.5.6) positioned above the waiting area and JBT arena (Fig. S3 in supplementary materials).

2.5. Recording of anticipation indicators

We video recorded hen behaviour continuously using one ceiling mounted camera during the 30-second waiting period prior to each of the six JBT testing sessions. The waiting period lasted 30 s as anticipation indicators peak in laying hens after a 22 s delay between a trained cue and subsequent reward presentation (Moe et al., 2009). The waiting period began when the sliding door between the transport and waiting boxes was fully lifted (transforming into the larger waiting area) and finished when the door to the JBT arena was fully lifted. The waiting area was visually divided into three equally sized zones: closest (zone 1), middle (zone 2), and farthest (zone 3) from the JBT arena.

We recorded anticipation indicators that laying hens show in response to conditioned cue (Davies et al., 2014; Moe et al., 2009, 2011, 2013; Wichman et al., 2012; Zimmerman et al., 2011), such as 'standing alert, 'alert head movements' (Table 2). In addition, we recorded latency to enter the JBT arena as the latency to approach a stimulus indicates motivation for that stimulus, with shorter latency for rewarding compared to aversive stimuli (Davies et al., 2014; Meagher and Mason, 2012). We also coded the frequency of transitions between different behaviours (henceforth 'behavioural transitions'), which increase during positive anticipation in many species, including laying hens (McGrath et al., 2016). The number of behavioural transitions was extracted from recorded measures of activity ('inactive still', 'active still', 'locomotion'). For all indicators, zone and body orientation in regard to the JBT arena entrance were recorded. From this, the duration of time spent within the different zones and orientated towards the entrance to the JBT arena were extracted. Due to inconsistencies in findings and lack of information, indicators of anticipation in laying hens so far cannot be distinguished based on whether they indicate positive versus negative anticipation. For this reason, we recorded the mentioned indicators (Table 2) without characterising whether they indicate positive or negative anticipation.

Table 2
Description of behavioural indicators of anticipation.

Indicators	Description
Indicators from which behavioural transition frequency was extracted	
Inactive still (s)*	One or both feet in contact with the ground (sitting or standing) with no forwards movements, with the body and neck in a neutral position (i.e., not shifted forwards or upwards) and with no additional movement of the wings or head such as pecking or preening or alert head movements.
Active still (s)*	One or both feet in contact with the ground with no forwards movements but with additional activity of the wings or head such as pecking or preening but excluding alert head movements.
Standing alert (s)*	One or both feet in contact with the ground with no forwards movements, with the front of the body shifted upwards and the neck stretched upwards.
Locomotion (s)*	Any forward or vertical movement of the hen's body such as walking, measured from the first step until three seconds have elapsed with no forward or vertical movement, or until the onset of a different behaviour.
Other indicators	
Alert head movements (s) *	Quick movement of the head in the vertical (raising/lowering) or horizontal (left/right movements) axes, which is not part of a peck, preen, ruffle or yawn, with each bout measured from the beginning of the first movement until three seconds have elapsed with no head movements, or until the onset of a different behaviour. Can be part of standing alert or locomotion behaviour.
Latency to enter (s)* *	Latency to enter the JBT arena, measured from the moment the access door lifts off the ground until the hen's entire body (including the tail) have passed through the door perimeter.

* Location and orientation of performance were recorded using three zones of equal size within the waiting area: zone 1 closest to the JBT arena entrance and zone 3 farthest, determined using the location of the front foot. Orientation recorded as 'towards' or 'away' in relation to the JBT arena entrance. The two orientations were complementary, with a body axis (head to tail) of ± 90 degrees from the arena entrance considered 'away' * * The location and orientation of the hen at the time the JBT door opens was recorded. Additionally, it was recorded whether the hen started walking immediately, i.e., within 3 s of the JBT door opening, or whether any other behaviour was performed before entering the arena (incl. stopping for more than 3 s).

2.6. Behavioural analysis

All behaviours (fear assessment and anticipator indicators) were analysed *post-hoc* on the video recordings by a single observer using BORIS Software (Friard and Gamba, 2016). As hens were marked for individual identification after treatment allocation, blinding of the hens' treatment during coding of the 2nd fear assessment was not possible.

Intra- and inter-rater reliability of all fear-related behaviours were assessed on 10% (n = 12) of all videos (four videos per test (OF, TI, HAT), balanced across fear assessment (1st and 2nd) and batch) with a second coder blind to both the treatment and fear assessment. For anticipation indicators, intra- and inter-rater reliability were assessed on eight randomly selected videos, and both coders were blinded to JBT test session number and the JBT test's outcome.

For all continuous indicators not showing 100% agreement, reliability was assessed using Intra-Class Correlation (ICC) estimates using the function icc of the package irr (version 0.84.1; Gamer et al., 2021) in R Studio (version 4.1.2; R Core Team; R Foundation for Statistical Computing, Vienna, 2020). As a general guide, the ICC reliability coefficient was considered poor when below 0.50, moderate when between 0.50 and 0.74, good when between 0.75 and 0.89, and excellent when higher than 0.9 (Harvey, 2021). The coefficients for both intra- and inter-rater reliability on fear-related behaviours revealed excellent reliability. For anticipation indicators, intra-rater reliability was either "good" (standing alert, locomotion, alert head movements) or 'excellent' (active, inactive, latency to enter). Inter-rater reliability was also either

'good' (standing alert, active, locomotion, alert head movements) or 'excellent' (latency to enter). The inter-rater coefficient for the indicator "inactive" was only "moderate," likely due to rare occurrence combined with relatively short duration of the recorded bout. Inter-rater reliability of the ordinal indicator 'avoidance distance' was assessed based on Cohen's Kappa. Whereas intra-rater reliability of the indicator 'avoidance distance' was excellent (100% consistency), only moderate inter-rater reliability was revealed. Detailed results of the intra- and inter-rater reliability are in (Table S1 in supplementary materials).

2.7. Sample collection and relative telomere length (RTL) analysis

We estimated RTL from whole blood DNA. Blood was collected from the brachial vein with a heparinised 1 ml syringe from all hens after the 1st fear assessment and prior to the start of the training in the trained hens. Hens were sampled again when 50% of trained hens had finished training and testing and underwent the 2nd fear assessment, i.e., the 2nd collection being 7 and 9 weeks after the 1st collection in batches 1 and 2, respectively. All hens within a batch were sampled on the same days for the first and second sampling, as age is known to affect RTL (Sohn and Subramani, 2014). For sampling, one person held the hens and the other person collected blood. The collector and handler were not involved in neither JBT training and testing nor fear assessment.

Samples were stored at − 80 °C until analysis. DNA was extracted prior to RTL analysis using a commercial kit (DNeasy Blood&Tissue Kit, Qiagen) and RTLs were obtained with a quantitative real-time PCR method (Cawthon, 2002; Criscuolo et al., 2009; Turbill et al., 2012) adapted for chickens. This (qPCR) method measures the relative amount of telomeric DNA (i.e., ratio of telomeric repeats versus a single copy gene control) rather than absolute telomere length (TL) per se. DNA from birds is known to contain interstitial telomere repeats away from chromosome ends, but relative TL measurement via qPCR has been well validated in birds and while the interstitial repeats may increase noise in the estimates, they too should act as a ROS trap and be a valid marker for oxidative stress (Criscuolo et al., 2009; Foote et al., 2013). We designed primers targeting a 171 bp sequence of GAPDH (segment of GenBank Acc. # NM_204305.2) as the non-variable copy number gene (non-VCN gene), which was selected for non-variability as described by Smith et al. (2011). Primer specificity was ensured by confirming the expected amplicon length via gel-electrophoresis. Forward and reverse telomeric primers were 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGG TT-3′ (tel 1b) and 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTA CCCT-3′ (tel 2b), respectively, and forward and reverse primers for the non-VCN gene (GAPDH) were 5′- TTG ACC TGA CCT GCC GTC TG-3′ (Ggall_GAPDH_F2) and 5′ CAG CAC CCG CAT CAA AGG TG-3′ (Ggall_GADPH_R2), respectively. Telomere and non-VCN gene PCRs were carried out in separate runs using 20 ng DNA per reaction in a Rotorgene Q (Qiagen, Germany) instrument. Primer pairs (Tel1b/Tel2b or Ggall_GAPDH_F2/Ggall_GAPDH_R2) were used in a concentration of 400 nM each, in a final volume of 20 µl containing 10 µl of GoTaq® qPCR MasterMix (Promega). PCR conditions for the telomere primers were 5 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C. For GADPH PCR conditions were 5 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 20 s at 62 °C and 20 s at 72 °C. PCR efficiencies and Ct-values (cycle threshold) were computed directly, without the use of standard curves with LinRegPCR software (2012.0) (Ruijter et al., 2009). LinRegPCR uses linear regression to estimate individual sample reaction efficiencies of non-baseline-corrected raw qPCR data (Ramakers et al., 2003) which gives lower but more accurate estimates of efficiency than standard curve based methods (Morinha et al., 2020; Spießberger et al., 2022). Mean qPCR efficiencies were 88.2% and 94.7% for the telomere and non-VCN gene reactions, respectively. To be able to compare RTL among plates, all telomere to non-VCN ratios were normalised to one individual (reference standard sample, RTL = 1), which was included in every qPCR run. RTL was calculated using a modified formula from Ruijter et al. (2009), where E is the qPCR efficiency and Ct the cycle threshold. The subscript ST refers to the telomere reaction of the standard sample, SC to the control gene reaction of the standard sample, T to the telomere reaction of the target sample and C to the control gene (GAPDH) reaction of the target sample: $RTL = (E_T^{CtT} / E_{ST}^{CtST}) / (E_c^{CtC} / E_{SC}^{CtSC})$. The intraclass correlation coefficient (ICC) was calculated as a measure of reliability within the runs, as suggested by (Koo and Li, 2016). ICC estimates and their 95% confident intervals for sample replicates were calculated in R Version 3.5.1 (R Core Team (R Foundation for Statistical Computing, Vienna, 2018)). Intra-rater ICC was calculated on all included data points based on a single-rating, absolute-agreement, 2-way mixed-effects model (ICC in library 'irr'; Gamer et al., 2021). Intra-assay ICC for Ct values for telomere assay was 0.995 ($p < 0.0001$, 95%;CI 0.993–0.996) and for GAPDH 0.997 ($p < 0.0001$, 95%;CI 0.993–0.998) showing an excellent degree of reliability.

The intra-assay coefficient of variation among replicates (intra-assay variation), an estimate of system precision, was further used to assess reproducibility. Mean intra-assay CV for Ct values of the non-VCN gene and telomere assay were 0.41% and 0.69%, respectively. A final melt step was included for each run to check for target specificity via unimodal melt dissociation peaks. The temperature ramping was set from 65 °C to 95 °C in 1 °C steps. For all assays, only the expected peaks for telomere and non-VCN genes were observed. A pipetting robot (Qiagility, Qiagen, Germany) was used for all assay runs to minimize pipetting errors and ensure consistency in reactions.

2.8. Statistical analysis

All models were fitted in R Studio (version 4.1.2; R Core Team; R Foundation for Statistical Computing, Vienna et al., 2021). Sample size was estimated so that there were at least 3–5 times as many observations (i.e., hens) than residual degrees of freedom (Gygax, 2013).

2.8.1. Fear-related behaviour

We first assessed the recorded indicators for correlations based on Spearman rank correlation tests (Table S2 in supplementary materials) using the function cor.test of the base R package stats (version 4.1.2; R Core Team; R Foundation for Statistical Computing, Vienna et al., 2021). None of the revealed correlation coefficients were high enough ($r > 0.8$; Field et al., 2012) to warrant exclusion from the final statistical analysis. We next fitted a suite of General/Generalized Linear Mixed Models (LMM/GLMM; Baayen, 2008); Table S3 in supplementary materials for an overview of all fitted models). Into the models, fear-related behaviour was considered a response indicator, treatment (JBT or control), fear assessment (1st or 2nd) and their interaction were included as fixed effects, with the interaction being of sole interest. To control for a potential batch effect, the batch (1 or 2) of each hen was also included as a fixed effect. Lastly, as we had repeated observations of the same subjects, individual hen ID was included as random intercept. As only the interaction effect of treatment and fear assessment was of interest, no reduced models lacking the interaction effect were fitted in the event that the interaction was not significant. Furthermore, as only one term was of interest (i.e., the interaction between treatment and fear assessment) there was no risk of 'cryptic multiple testing' (Forstmeier and Schielzeth, 2011) and thus no full-null model comparisons were required.

Assumptions of residual normality and homoscedasticity were assessed for LMMs through visual inspection (Quinn and Keough, 2002). Collinearity between predictor indicators was assessed for all models by determining Variance Inflation Factors based on models lacking the interaction using the function vif of the package car (version 3.0–12; Fox and Weisberg, 2019). No issues of collinearity were detected for any of the models (maximum Variance Inflation Factor: 1.086; Quinn and Keough, 2002). For Cox regression models, assumptions of proportional hazards were assessed based on the scaled Schoenfeld Residuals, using the function cox.zph of the package Survival. The presence of

overdispersion and model stability were also assessed. Model stability, i.e., a test of influential cases in the dataset, was assessed on the basis of DFBeta-Values by dropping one subject from the dataset at a time and comparing the derived estimates from models fitted using the resulting subsets with the original model estimates (Nieuwenhuis et al., 2012). This revealed a number of models to be of only moderate stability (i.e., OF: steps (no); HAT: First step (s); TI: attempts (no), duration of tonic immobility (s). Poor model stability suggests the presence of influential cases and introduces uncertainty into the model, thus preventing the ability to make strong claims based on the model outputs (Nieuwenhuis et al., 2012). Estimates and p-values for individual effects in LMMs were obtained using Satterthwaite approximation for a model based on REML (Luke, 2017). For all other models, they were derived using likelihood ratio tests with the function drop1 with argument 'test' set to 'Chisq' (Barr et al., 2013). For any significant interaction effects, *post-hoc* analysis using function emmeans of the package emmeans (version 1.7.2; Lenth, 2022) was performed to determine which contrasts were significant, with p-values Holm-corrected for multiple testing across all contrasts. Lastly, confidence intervals of model estimates and the fitted models were derived using the function 'confint' or via means of a parametric bootstrap (N = 1000).

The indicators 'jumps', 'vocalisations', 'touching human' and ADT were excluded from statistical analysis due to infrequent observation (i.e., jumps: no jumps observed; vocalisations: a total number of eight tests out of 60 in which a hen vocalised; touching human a total number of 11 tests out of 60 in which a hen touched a human) or little change in response between fear assessments and treatments (i.e., avoidance distance).

2.8.2. Telomere shortening

We analysed the data with the ANOVA and RTL shortening (i.e., difference in RTL before and after separation) as the response indicator and treatment and batch as fixed effects.

2.8.3. Anticipation indicators

We first assessed the recorded and extracted anticipation indicators for correlations in order to reduce the number of indicators included in the model to fewer uncorrelated indicators and to reduce model complexity. Relationships between indicators were assessed based on Spearman rank correlation tests (Table S4 in supplementary materials) using the function cor.test of the base R package stats (version 4.1.2; R Core Team;R Foundation for Statistical Computing, Vienna et al., 2021). None of the correlation coefficients were above 0.8 (Field et al., 2012), thus, all anticipation indicators were included in the final model.

The proportion of Go-responses during JBT tests was included into the model by using a two-columns matrix with the number of Go and No-Go-responses during ambiguous trials per hen per JBT test session included as the response indicator (Baayen, 2008). We fitted a Logistic Generalized Mixed Model (GLMM; Baayen, 2008), into which we included all anticipation indicators as well as the JBT test session number (1−6) and the interaction between both anticipation indicators and the JBT test session number, as fixed effects. We included the interaction as we expected that a learning effect might occur, with anticipation indicators influenced by the session number. Additionally, batch (1 or 2) was also included as a fixed effect. As random intercepts, we included subject and the number of sessions needed for hens to reach final learning criteria. As an overall test of the effect of the included predictors, and to control for risk of 'cryptic multiple testing' (Forstmeier and Schielzeth, 2011), a full-null model comparison was performed, with a null model lacking the anticipation indicators and JBT test session number but including batch and the same random effects structure, using a likelihood ratio test (Dobson, 2002). Tests of the individual fixed effects predictors were derived with likelihood ratio tests using the function drop1 with argument 'test' set to 'Chisq' (Barr et al., 2013). The model was fitted in R Studio (version 4.1.2; R Core Team; R Foundation for Statistical Computing, Vienna et al., 2021) using the function glmer of the package lme4 (version 1.1.28; Bates et al., 2015). Prior to fitting the model, we z-transformed anticipation indicators (to a mean of 0 and a standard deviation of 1) to increase the likelihood of model convergence (Schielzeth, 2010). The presence of influential cases and model stability was assessed and revealed the model to be of good stability, except with regard to the effect of level 5 (i.e., JBT test session 5) of the indicator JBT test session number, which was largely unstable. No issues of collinearity were detected with a maximum Variance Inflation Factor of 1.083 (Quinn and Keough, 2002). Finally, the presence of overdispersion was assessed, revealing a dispersion parameter of 0.66, indicating that the model was moderately underdispersed. The sample for this model consisted of 81 observations from 14 hens.

3. Results

3.1. Fear-related behaviour

There was a significant interaction between treatment and fear assessment time point on hens' latency to first step in the OF test (t= 3.129, df=28, P=0.012; Fig. 1), with trained hens showing a longer latency to first step from the 1st to the 2nd fear assessment (i.e., after receiving training; t=-2.827, df=28, P=0.017), while control hens showed a reduced latency to first step, but this was not statistically significant (t=1.559, df=28, P=0.13). No other significant interaction effects were found. In the 2nd fear assessment, both groups spent less time close to the test-human in the voluntary approach test, had a similar ADT and showed a marginal increase in proportion of time spent showing exploration compared to the 1st fear assessment (descriptive results in Table 3). Full results of all statistical models are available in Table S5 in the supplementary materials.

3.2. Telomere shortening

There were no differences between treatments on telomere shortening *(f=1.043, df=1, P = 0.316)* and a tendency for accelerated telomere shortening in batch 2 hens compared to hens from batch 1 *(f=3.180, df=1, P = 0.085)*.

Table 3
The mean ± SEM for measured fear assessment indicators during the first and second fear assessments, including p-values for the interaction effect between treatment and timepoint. Open field test (OF, 2 min), Human approach/avoidance test (HAT, 2 min), Tonic immobility test (TI). Values are shown for both treatments (control, trained) at the first and second fear assessments. Bolded and italicised p-values indicate significant interaction effect between treatment and fear assessment.

Test	Indicator	Control		Trained		
		First	**Second**	**First**	**Second**	**P**
OF						
	Latency to first step (s)	***33.66 ± 8.74***	***18.87 ± 5.60***	***12.01 ± 3.16***	***30.89 ± 9.07***	***0.012***
	Steps (No.)	20.36 ± 3.92	25.33 ± 3.20	20.36 ± 2.75	15.50 ± 3.82	0.504
	Exploration (prop.)	0.01 ± 0.005	0.05 ± 0.015	0.02 ± 0.019	0.03 ± 0.013	0.504
HAT						
	Latency to first step (s)	20.27 ± 8.54	14.01 ± 3.12	10.91 ± 3.68	38.90 ± 11.88	0.154
	Proximity (prop.)	0.29 ± 0.08	0.17 ± 0.06	0.29 ± 0.09	0.21 ± 0.08	0.951
TI						
	Attempts (No.)	2.44 ± 0.35	2.00 ± 0.30	2.29 ± 0.32	2.00 ± 0.36	0.835
	Duration to right (s)	263.32 ± 49.32	173.92 ± 47.04	293.80 ± 58.63	362.22 ± 68.21	0.154

3.3. JBT outcome and anticipation indicators

In total, 14 hens finished the full training protocol and were tested in the JBT, with training requiring an average of 35.36 sessions (± 7.75 SD). Hens completed on average 17.43 (± 4.3 SD) sessions for habituation, 8.29 (± 3.02 SD) for shaping, 3.43 (± 1.99 SD) for left-right discrimination, and 6.53 (± 2.64 SD) for Go/No-go discrimination. We checked for a learning effect (i.e., whether Go responses increased across test sessions) via visual inspection (Fig. S3 in supplementary materials) (Fig. 2).

Trained hens' Go responses showed a monotonic graded response with regard to the different goal-holes, indicating that hens interpreted the ambiguous goal-holes in relation to the reference goal-holes (Fig. 3 below; Hintze et al., 2018). Overall, trained hens showed an average proportion of Go responses to ambiguous cues of 0.7 (± SE 0.04), including one hen that showed 0.28 and another showing 0.5 proportion of Go responses to ambiguous cues, and the rest of hens showed a greater than 0.5 proportion of Go responses.

There was no effect of the anticipation indicators on the JBT outcome (full-null model comparison, likelihood ratio test: $\chi 2 = 117.7835$, $df=45.35$, $P = 0.54$) and no significant interaction effects between any of the anticipation indicators and JBT test session number, or significant main effects in a reduced model lacking the interactions ($P > 0.05$). Descriptive results of anticipation indicators shown before each of the JBT test sessions and full results of all statistical models are available in Table S6, Table S7, and Table S8 in the supplementary materials.

4. Discussion

We expected that because hens get habituated to stressful elements in the training protocol the JBT training would be enriching and improve hens' welfare. Contrary to our hypothesis, with the exception of latency to step, we did not observe a reduction in fear-related behaviour or deceleration in telomere shortening in trained hens compared to control hens.

4.1. The effect of training on fear-related behaviour

Various fear assessment tests (TI, HAT, OF) assess different aspects of fear (i.e., fear of predation, humans and novelty) as well as social motivation (Forkman et al., 2007). Thus, results from these fear assessment tests should be interpreted together for an adequate assessment of general fear. In our study, there was no consistent evidence across fear assessment tests that training and testing in the JBT affected fear-related behaviour. Five out of seven indicators showed a similar effect of increased fear-related behaviour in the 2nd fear assessment compared to the 1st in the trained hens, and either no change or decreased fear-related behaviour in the control hens, but only one indicator (first step in the OF) significantly differed according to the training experience. As activity-related indicators in the fear assessment tests can be influenced by numerous internal factors (e.g., fear of novelty, social motivation, exploration; Nielsen, 2022), we are unable to make strong claims regarding fear based on this one indicator alone. Furthermore, hens were not allocated to treatments based on this indicator and the treatments differed numerically in the 1st fear assessment which could have biased the results.

Overall, our hens expressed lower (OF) to similar intensity of fear-related behaviour (HAT, TI) compared to other studies (Bari et al., 2021; Dumontier et al., 2022; Suarez and Gallup, 1981). Thus, together with a low number of vocalisation and a higher number of steps taken compared to other studies in the OF test (Campbell et al., 2019) indicates that our hens likely experienced low levels of fear in the OF test, which is thought to assess fear of novelty (Jones and Waddington, 1992; Moriarty, 1995; Suarez and Gallup, 1981) and social motivation (Suarez and Gallup, 1983, 1981). Hens in our study showed a similar latency to first step in the presence of a human as in other studies (for example, Larsen et al., 2018). This indicator is not affected by regular handling nor visual contact with humans (Jones, 1993) and may thus indicate fear of the novel environment in the HAT rather than fear of humans specifically. Distance to a human likely indicates only the quantity and not the valence of experience with humans as there is no difference in the distance to a human in chickens exposed to rough or gentle handling or those that only had visual contact with humans (Jones, 1993). Other studies confirm that visual contact reduces fear of humans in commercially kept chickens (Taylor et al., 2022; Zulkifli et al., 2002). It is difficult to compare the amount of contact with a human across studies as it involves not only study-related (e.g. training and testing) related contact, but also regular handling and care. Our hens likely had more frequent experience with handling due to regular collecting of the training hens. Whereas hens in commercial systems likely have longer visual contact with humans during their everyday flock-inspections due to larger flock sizes.

Decreased duration of tonic immobility and increased number of attempts to induce it are considered indicators of fear of predation

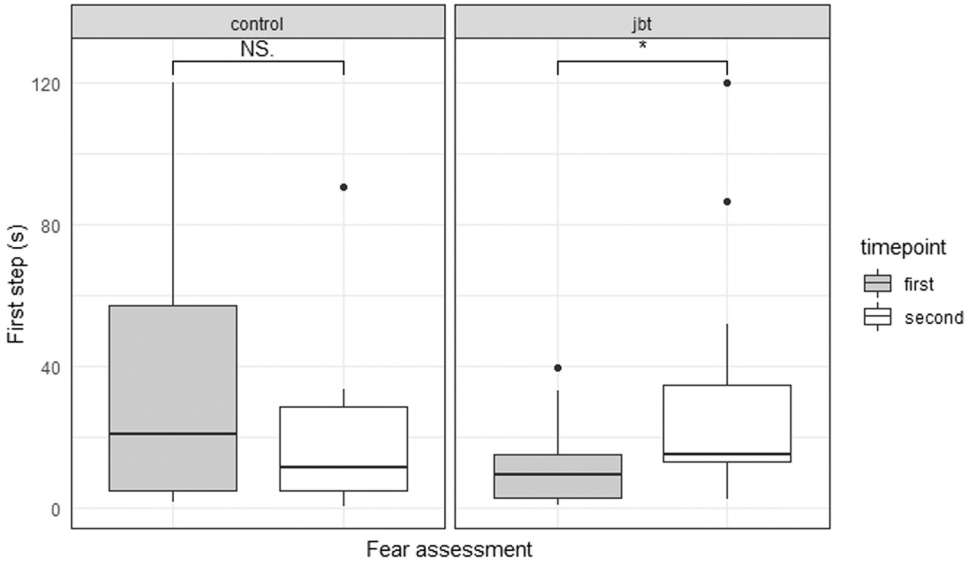

Fig. 2. Latency to first step in the open field test. Shown separately for control and trained (jbt) hens. Significance level for the contrast between fear assessment timepoints indicated above; * = P < 0.05.

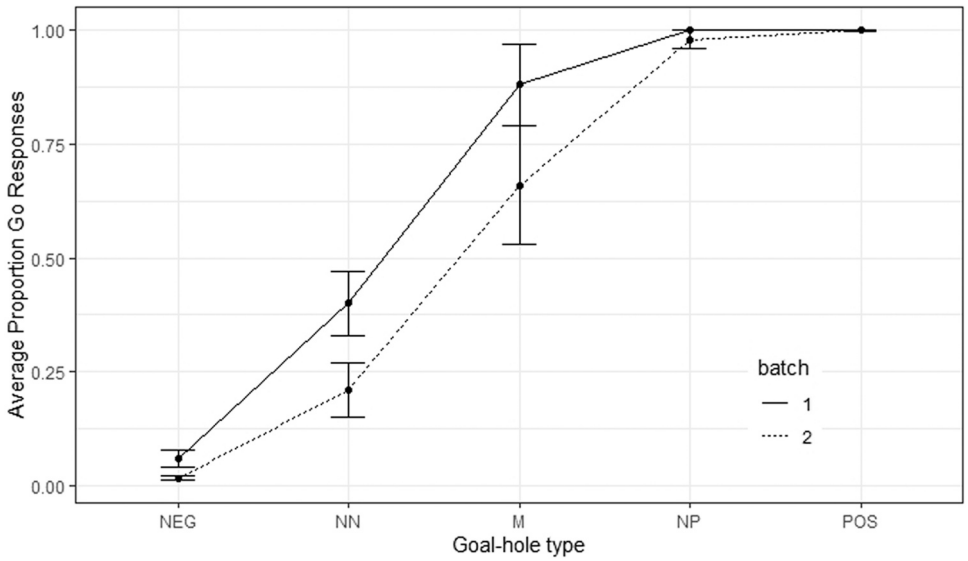

Fig. 3. Average proportion of Go responses (mean ± SE). Shown for both batches for each goal-hole type (NEG=Negative, NN= Near Negative, M=Middle, NP=Near Positive, POS=Positive) across all six Judgement Bias Task test sessions.

(Gallup et al., 1971a) and humans (Gallup Jr et al., 1972; Jones and Waddington, 1992). Compared to other studies, our hens required a similar (Campbell et al., 2019) or greater (Jensen et al., 2005) number of attempts to induce TI and showed a similar or longer duration of TI (Campbell et al., 2019; Jensen et al., 2005; Larsen et al., 2018; Wichman et al., 2012), which may be due to differences in a technique used to induce TI and duration of restraint (Gallup et al., 1971b).

We performed more habituation sessions compared to other studies in laying hens (de Haas et al., 2017; Ross et al., 2019) and all but one hen successfully habituated to training and the related stressors. Moreover, hens voluntarily entered the JBT arena and actively initiated trials, indicating that JBT sessions may have provided hens with a positive experience. Thus, it is surprising that fear-related behaviour was not affected. No difference in the HAT and TI could be expected as behaviour in both tests is influenced by hens' fear of humans (Forkman et al., 2007; Jones and Waddington, 1992) and both treatments may have habituated to humans due to daily interactions. Nevertheless, trained hens experienced slightly more handling compared to control hens and were thus expected to show reduced distance to a human in the HAT.

It is possible that the lack of strong effect across the fear assessment tests could be explained by an expectation mismatch. Hens likely learnt to anticipate JBT sessions after receiving almost daily training, as observed in dolphins (Clegg and Delfour, 2018) and seals (Podturkin et al., 2022), and may have had an expectation of being transported to the JBT arena. Being transported instead to the fear assessment arena could have caused a discrepancy between what they anticipated versus what occurred. This expectation mismatch may have in turn influenced hens' behaviour during fear assessment, masking the effects of JBT training on behaviour.

Studies showing positive effects of cognitive enrichment on fear-related behaviour usually provided no additional enrichments (Oesterwind et al., 2016; Puppe et al., 2007; Zebunke et al., 2013). In contrast, we housed all hens in enriched housing. Additionally, all hens were exposed to visual human contact during daily pen disturbances for training and husbandry procedures. Environmental enrichment and human contact reduces fear-related behaviour in domestic chickens (Dumontier et al., 2022; Jones and Waddington, 1992; Ross et al., 2019). Thus, additional effects of cognitive enrichment provided by JBT sessions on fear-related behaviour may have been minor. To disentangle this potential confounding effect, it would be interesting to study hens housed in enriched versus non-enriched environments that either undergo training or not.

We assessed fear-related behaviour in hens experiencing all elements of training, i.e., handling, transport, social isolation, novelty, and training. Although most hens satisfied the habituation criteria and only those underwent the 2nd fear assessment, it is possible that certain components of training remained somewhat negative. Previous studies showing positive effects of cognitive enrichment in other species (Oesterwind et al., 2016; Puppe et al., 2007; Zebunke et al., 2013) and of environmental enrichment in chickens (Jones and Waddington, 1992; Tahamtani et al., 2020) provided the studied enrichments in the home pen, avoiding social isolation and giving animals control over interacting with the enrichment. Future studies could employ additional control groups (i.e., handling only, handling and transport without training) and assess the effects of training within the home pen or automated training; however, this was beyond the scope of our study.

In our study, hens took on average five weeks to reach full learning criteria and needed another week for JBT testing. In comparison, studies showing positive effects of cognitive enrichment usually provided enrichment for longer periods (e.g., 8 weeks in pigs (Zebunke et al., 2013) and goats (Oesterwind et al., 2016) and 14 weeks in pigs (Puppe et al., 2007)), with effects on fear increasing with time (Puppe et al., 2007). Moreover, the effect on behaviour is likely greater during the early developmental period than in adulthood (Brantsæter et al., 2016). Thus, it is possible that another task that demands longer training, or the same task completed with younger animals may have led to a positive effect on fear-related behaviour.

Fear assessment tests may be less effective in repeated assessments (Jones, 1989). The design of our study required hens to be tested twice in the fear assessments so that each hen could act as her own control. As a result, however, novelty of the fear assessment procedure may have been reduced in the 2nd assessment, which could have affected hens' behaviour (Nielsen, 2020) and may have influenced the likelihood of detecting an effect. It is possible that an effect would have been found had hens been assessed only after JBT training versus no training, however, not performing the 1st fear assessment would have reduced our ability to interpret any significant results in relation to the treatment itself.

4.2. *The effect of training on telomere shortening*

Contrary to our prediction, telomere shortening was not affected by the JBT training. Stress-related differences in telomere shortening due to being housed at high stocking densities were successfully detected after

two weeks in young broiler chickens (Beloor et al., 2010) and four weeks in laying hens of stress exposure when measured with a more sensitive method (i.e. fluorescence in situ hybridization; Sohn et al., 2012) compared to the qPCR (Nettle et al., 2021). In young chickens, the qPCR method was able to detect differences in telomere length after four weeks of stress (corticosterone administration) (Badmus et al., 2021). We could not find any study using qPCR in adult hens to measure telomere length, but the interval between the sampling time-points in our study (i.e., seven (batch 1) and nine weeks (batch 2) was likely long enough to detect any training-related effects on telomere shortening. Although qPCR is a standard method to measure telomere length in a variety of species due to its ease of application and reduced DNA concentration requirement (Bateson, 2016), it is susceptible to a large measurement error, particularly for dual timepoint tests (Nettle et al., 2021). Hence, a more precise method such as a gold-standard telomere restriction fragment (TRF) analysis would likely increase the statistical power, though it was not possible to establish TRF for the current study.

Results from telomere shortening do not provide information on how various elements of training affected hens' welfare. Interactive effects of negative experience related to social isolation and handling that accelerate telomere shortening (Chatelain et al., 2020; Mathur et al., 2016) and positive experience due to cognitive enrichment that decelerate telomere shortening (Schutte et al., 2020) are likely, but could not be explained with our design. We observed a tendency for a batch effect and batch 2 hens showed accelerated telomere shortening compared to batch 1 hens. Hens in batch 2 also had no darkness in the first 2 weeks of the study, which is a known stressor (Campo & Dávila, 2002), and is a possible reason for accelerated telomere shortening. However, batch 2 consisted of younger hens than batch 1. Telomeres shortening is faster in young animals than older individuals before sexual maturation, but telomere shortening is not necessarily related with age in adult animals as seen in birds (Hall et al., 2004) and mammals (Seeker et al., 2018). At the start of the study, batch 1 hens were at the peak of lay and batch 2 at the beginning, indicating sexual maturation. It has not yet been identified at what developmental stage telomere shortening stops relating to age in domestic chickens, thus it is possible that age affected the batch-related tendency for telomere shortening. Time between sample collections was longer in batch 2 than in batch 1, which could also have affected the results.

4.3. The relationship between anticipation of JBT test sessions and the JBT test outcome

Laying hens show anticipation indicators in response to recurrent events (Davies et al., 2015; Moe et al., 2013, 2011, 2009; Zimmerman et al., 2011). We hypothesised that anticipation indicators shown prior to the JBT test session related to hens' experience in past JBT sessions and would in turn affect the JBT outcome. However, we found no support for our hypothesis.

An animal's mood state, i.e., their long-term affective state, influences decision-making processes when tested in a JBT (Lagisz et al., 2020; Neville et al., 2020). However, the assessment of mood states with a JBT may be confounded by short-term emotions induced prior to (Doyle et al., 2020; (Salmeto et al., 2011), or even during testing, challenging the robustness of the method to assess mood states specifically (Mendl et al., 2010). As we found no effect of anticipation on the outcome of the test itself, this suggests that the JBT may be robust against the emotions associated with anticipation of JBT sessions. However, as anticipation indicators are directed towards attaining a particular outcome (Krebs et al., 2022), it is possible that we assessed anticipation to enter the JBT arena and not for engagement with the test or approaching the cues.

Variation in anticipation indicators before the JBT test sessions as well as variation in the JBT outcome were low, which may have affected our ability to see any effect of anticipation on the JBT outcome. This low variation may be explained by the lack of affect manipulation in our study. However, other studies have shown that individuals kept under the same conditions vary with respect to JBT outcomes, probably due to individual differences in how the physical and social environments are perceived (de Haas et al., 2017; Hernandez et al., 2015; Hintze et al., 2018). Introducing an additional welfare manipulation, such as differing housing conditions, could increase the variation in the affective state and anticipation of JBT sessions.

5. Conclusion

Contra to our hypotheses, we found limited evidence for an effect of training for a JBT on fear-related behaviour and no effect on telomere length. This suggests that, under enriched housing conditions, the same animals can be used in the JBT and assessment of fear-related behaviour and telomere length. However, the enriched home environment might have minimised the enrichment effect of the training and thus the effect on fear-related behaviour and telomere length. Future research should study the effect of training on animals in various conditions (e.g., enriched vs. barren housing) as well as separate elements of training for behavioural tests on welfare.

We also found no support for our hypothesis that behavioural indicators of anticipation would predict the JBT outcome, indicating that the JBT outcome may be robust to short-term affective states related to anticipation of the task. However, we observed a low level of variation in anticipation indicators and the JBT outcome, which could have led to a low power in detecting an effect.

CRediT authorship contribution statement

Johanna Neuhauser: design of the study, acquisition of data, analysis and interpretation of data, drafting and revising the article. **Sara Hintze:** design of the study, interpretation of data, revising the article. **Steve Smith:** interpretation of data, revising the article. **Jean-Loup Rault:** design of the study, interpretation of data, revising the article. **Janja Sirovnik:** conception and design of the study, acquisition of data, analysis and interpretation of data, drafting and revising the article.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2023.105996.

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