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The effects of transport of 18-day old hatching eggs on physiology and behaviour of slow growing broiler chicken

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ABSTRACT

Incubation and hatching commonly takes places at hatcheries, separate from the grow-out facilities where broiler chicks are raised. This means that chicks are sorted and transported immediately after hatch, during which time they typically do not have access to feed and water, and are subjected to transport stress. Recently, innovative housing systems are being developed in which fertilised eggs are transported on embryonic day 18 (E18) from the hatchery to the grow-out facility, where they hatch on day 21. In chicken, the hypothalamic–pituitary–adrenal (HPA)-axis becomes functional around embryonic day 14–16. It is therefore conceivable that transport of eggs at E18 may lead to a stress response in the chick embryo. Exposure to prenatal stress may affect the coping capacity of the individual and negatively impact its further development. We investigated whether prolonged transport on E18 has effects on the development of a slow growing broiler chicken strain (Hubbard JA257). E18 eggs were transported for either 41 min (short transport, ST) or 219 min (long transport, LT). Transportation significantly increased embryonic heart rate after ST. This increase continued during an intermediate measure at 120 min. The increased embryonic HR then remained high at measurement immediately following LT. We did not find effects of prolonged transport on behavioural parameters measured in the juvenile chicken in the tonic immobility and open field test. Concentrations of feather corticosterone as well as faecal corticosterone metabolites did not differ on postnatal day 36. We showed that transport leads to an autonomic stress response in chicken embryos at E18, but that this elevation had no further effects on other indicators of prenatal stress. Nevertheless, our results emphasise that transport of incubated eggs should be as refined as possible to minimise the exposure to stress.

1. Introduction

In conventional broiler chicken farming, broiler chicks hatch in a hatchery and are then transported at 1 day of age to a grow-out farm (van de Ven et al., 2009). During the transportation from the hatchery to the grow-out facility, the one-day old chicks are exposed to various stressors, including fluctuating temperatures, low air quality, motion, noise and social disruption (Mitchell and Kettlewell, 1998; Khosravinia, 2015). This results not only in behavioural and physiological responses with potential long-term effects (Janczak et al., 2006; Marasco et al., 2012; Ahmed et al., 2016) but also in mortality attributable to transport stress (Bayliss and Hinton, 1990; Mitchell, 2009; Vieira et al., 2019).

One way to potentially avoid this transportation stress is on-farm hatching. In this system, the eggs are transported to the grow-out farm on day 18 of development (E18), and thus hatch in the same location as the chicks are subsequently reared (e.g. Patio or X-treck Vencomatic) (de Jong et al., 2018). A number of recent studies demonstrated welfare improvement in chicks following on-farm hatch compared to transportation as day-old chicks. On day 0 post-hatch, increased hatchability and body weight have been reported in chicks hatched on-farm compared to in a hatchery (van de Ven et al., 2009; de Jong et al., 2019), although this may come at a cost to chick quality as determined by naval and hock scores (de Jong et al., 2019, 2020; Souza da Silva et al., 2021). Broilers hatched on-farm showed decreased mortality rates

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at day 7 (van de Ven et al., 2009), as well as less footpad dermatitis and better litter quality in on-farm hatch chicks at 21 days of age (de Jong et al., 2019). On-farm hatch does not seem to affect behavioural measures of stress or fear in a non-organic farming setting (Giersberg et al., 2020, 2021), but has been reported to reduce fear early in life in organically housed poultry (van de Ven et al., 2009).

Collectively, these studies show that both the performance and the welfare of broilers may be positively influenced in on-farm hatching systems. Nonetheless, an important aspect that is frequently overlooked is that transport still takes place, but earlier. Rather than transportation of day-old chicks, in on-farm hatch to embryos are transported *in ovo* at E18. Parts of the hypothalamic- pituitary- adrenocortico (HPA) axis, which plays a crucial role in stress responses, are already developed in very early embryogenesis in chickens. This is visible as early as day E7 (early development in the median eminence, see Daikoku et al., 1974) and the HPA-axis is largely functional by day E14 (Jenkins and Porter, 2004). This development of the HPA-axis is well before transportation in on-farm hatching systems (usually day E18), making it possible if not likely that the chick embryos are responsive to stressors at transport such as vibrations or noise. Studies of effects of vibration *in ovo* reminiscent of transportation at day E1 have shown that vibrations during early embryogenesis (Donofre et al., 2017) or chronically between day E0 and day E15 (Shannon et al., 1994) can reduce the percentage of chicks hatching. Exposure to sound *in ovo* may also affect chick development, possibly to the benefit of hatchability in some cases of chronic sound exposure continuously throughout incubation (Donofre et al., 2020) but with long-term effects on behaviour seen in quail following chronic noise exposure *in ovo* (Mezrai et al., 2022). The auditory system in chicks is developed and responsive to a mature hearing range by day E16-E18 (Jones et al., 2006), making it likely that embryos can respond to transportation noise at day E18 as well.

There is a knowledge gap in the effect of prenatal transport, especially at E18, on chicken physiology and later development. Therefore, the present study focuses on the physiological and behavioural effects of transportation of broiler chickens *in ovo* at E18 for a short amount of time (41 min) or a long amount of time (219 min). We measured effects both in the short term by measuring heart rate of the embryo *in ovo*, and in the long term by measuring corticosterone (CORT) in feathers at day 36 post-hatch, and CORT metabolites in faeces, growth rates (weight and tarsus), and performance in fear-related behavioural tasks (open field and tonic immobility) in juvenile chicks followed through to 44 days post-hatch. Compared to short transportation, long transportation is hypothesised to have more effect on physiological parameters acutely (increased HR), stress physiology on the long term (increased CORT in feathers and faeces) and adversely impact growth (body weight and tarsus length). We further hypothesise that longer transportation will lead to more fearful behaviours in the open field and tonic immobility tests conducted in juvenile chicks.

2. Materials and methods

2.1. Ethical approval and animal welfare

This study protocol (WP10818–2019–1) was reviewed and approved by the local Animal Welfare Body of Utrecht University, the Netherlands, according to the European Directive 2010/63/EU and the Dutch Experiments on Animals Act (WOD) as amended on December 18, 2014.

All procedures were executed in accordance with the national guidelines on animal care. To monitor the health of the chickens, they were weighed twice a week and a general health check was done once each week. During health checks, all chickens were inspected for behaviour, injuries and illnesses (see Appendix A). If a chicken showed compromised health, the individual was checked daily. If the chicken did not show any improvement within three days, the case was discussed with a veterinarian specialised in poultry to define whether a humane endpoint had been reached. One male individual was euthanized in the second week, as the chick had lost weight for two measurements in a row and showed a weak body condition. The autopsy did not indicate that there was an effect of treatment.

2.2. Animals, transport treatment and embryonic heart rate measurement

In May 2019, 90 hatching eggs of slow-growing broiler chickens (strain Hubbard JA257) were collected at a commercial breeding facility (Morren BV, Lunteren, The Netherlands) on E18. All eggs were placed in a truck with a controlled environment (Heering BV, Vaassen, The Netherlands). Temperature was kept at approximately 33 ºC ($\pm$ 1ºC) during transport. The eggs were distributed across four trays and allocated in a random manner to the two treatment groups: short and long transportation. The short transportation (ST) group was brought directly to the research facility (1 trip, 35 km, 41 min, Faculty of Veterinary Medicine, Utrecht University), whereas the long transportation (LT) group was transported approximately 5 times longer (5 trips, 175 km, 219 min).

Before, during and after the transportation, heart rate (beats per minute, bpm) was recorded for 12 ST eggs and 12 LT eggs. Three eggs were chosen randomly on each of the four transportation trays. The same 24 focal eggs were tested for HR before, during, and after transportation. Two eggs of each treatment were placed simultaneously in one of four digital egg monitors (Buddy™, Vetronic Services, UK) and heart rate was noted every 15 s for 2 min, following the protocol by Lierz et al. (2006). Heart rate was recorded immediately prior to departure from the breeding facility (HR 0) and immediately upon arrival at the research facility (HR1). The ST eggs were then removed from the truck and placed in one of the two pre-warmed incubators. The incubator temperature was set at 36ºC (lowest possible temperature) to keep the difference between the temperature in the truck and in the incubators at a minimum. The LT eggs were transported back and forth to the breeding facility two more times, totalling a route five times longer than the ST eggs. The heart rate of the LT eggs was measured again after returning to the research facility again (HR3 ca. 120 min) and after the final return (HR5; Fig. 1).

After the final return, ST and LT eggs were newly divided over six trays across the two incubators (37ºC, 70% relative humidity), balanced for treatment. Each tray was divided into 15 compartments (3 $\times$5 cm per compartment) and eggs were placed into the compartments to be able to trace back the hatched chick to the egg. Three eggs showed cracks in their shell, which were covered with parafilm during incubation.

2.3. Hatching and animal identification

Hatching was checked on E19 (no chicks hatched). On E20, 12.00 (noon), 44 chicks had hatched, and by 16.00, another 15 chicks had hatched. The remaining 30 eggs hatched by 09.00 on day E21, with the exception of one egg, that had been covered with parafilm on E18 due to external shell damage; the chick was manually released from that egg. All chicks were taken out of the incubator as soon as their feathers had dried. The chicks were weighed, ringed, and placed in one of the four pens. All eggs included in the experiment hatched (89 naturally, 1 manually). Chicks were not vaccinated, nor beak trimmed.

For the first 2–3 days, the chicks were marked with coloured leg rings to allow individual identification. To further aid individual identification, the feathers of the chicks in pens 1 and 2 were given a unique pattern using either blue or green non-toxic paint on day two. The chicks in pens 3 and 4 were picked up and handled in a similar way but without painting the feathers. On day five, the chicks in pens 3 and 4 were painted and those from pens 1 and 2 were handled. On day eight, the leg rings were removed from all chicks. On day 44, at the end of the experiment, the chicks were euthanized by cervical dislocation. Sexes were determined by visual characteristics and confirmed by PCR

analyses of the sex-linked genes (see Section 2.7).

2.4. Animal husbandry

After hatching, as soon as the feathers had dried, the chicks were divided over four pens in a balanced manner, to achieve equal numbers per pen (Table 1).

The pens measured 4 × 2×2.2 m and were located next to one another. The wire mesh walls that separate the pens were covered with anti-root fabric to prevent visual contact between chickens from adjacent pens. Auditory contact remained possible. Each pen had an automatic drinking system, two feeders, and heating lamps and plates, and the floor was covered with wood shaving and straw. Water and food (De Heus voeders BV: starter (Opfmeel F1 AC zakgoed (starting day 1), Foktoom SLF F2 kruimel (starting day 15), Finisher: ABZ Diervoeding, 94017 V/K ST/GR GD (starting day 32)) were available ad libitum; diet formulation included in Table 2. When the feed was changed, a transition period of at least 3 days was applied, in which the chickens received a 50:50% mix of the old and new feed. Availability of food and water and the cleanliness of the pens were checked every morning.

Light was provided through large skylights and windows. At the start of experiment sunup was at 05:45 and sundown at 21:27; at the end of experiments sunup was at 05:20 and sundown at 22:04. Temperature and humidity were noted daily to ensure a suitable and similar environment in all pens. In the first four days a low relative humidity between 20% and 30% was measured. Therefore, to increase humidity, water buckets were placed outside the pens. After that, the relative humidity was kept at 61.4% ± 15.6%. The temperature was maintained between a mean minimum temperature of 20.8ºC and a maximum temperature of 27.7ºC using heating lamps. As enrichment, perches were installed and a hay bale was added to each pen on day 11.

2.5. DNA sex determination

Over the course of week 5 and 6, the sex of all chickens was estimated based on secondary physical characteristics (comb and wattles). During handling in these two weeks, shed feathers were collected to use for DNA extraction and sex determination by amplification of the sex-linked genes CHD-W and CHD-Z (Sulandart and Zein, 2012). The feathers were stored in paper envelopes and at room temperature in a dark cabinet. One to two mm of the calamus were cut from one or two feathers of an individual and transferred to a tube. 100 μL 5% Chelex solution (Biorad, USA) was added to each tube to extract DNA. The supernatant was used for DNA amplification by PCR using the primer set 2550 F/2718 R. The product was made visible by gel-electrophoresis yielding two bands for females (WZ) and one band for males (ZZ).

2.6. Body mass, tarsus and health scores

Starting from hatch, body mass was recorded twice a week, on Mondays and Fridays. Tarsus length, as an indicator of structural body size (Senar and Pascual, 1997), was measured to the nearest 0.01 mm on day 1 and day 44 using a digital calliper. The average of three measurements was taken for statistical analyses.

At age 36d, all chicken were carefully examined and given a score for plumage cleanliness (0−3), breast discoloration/blisters (0−2), foot pad lesions left and right (0−4), hock burns (0−4), and skin lesions (0−2) (scores 0 being the least/best/most clean, highest scores being the worst) (Giersberg et al., 2021).

Table 1
Overview of individual chickens in each pen (ST = short transport, LT = long transport).

Pen	Number of chicks	ST individuals	LT individuals
1	22	10 (5 f, 5 m)	12 (5 f, 7 m)
2	22 (1 chick euthanized in week 2)	11 (4 f, 7 m)	11 (6 f, 5 m; 4 m after week 2)
3	22	12 (5 f, 7 m)	10 (6 f, 4 m)
4	24	12 (6 f, 6 m)	12 (5 f, 7 m)
TOTAL	90 (89 after week 2)	45 (20 f, 25 m)	45 (22 f, 23 m; 22 m after week 2)

Table 2
Specification of the diet formulation used during the study.

Day	Nutritional information (%)	Name and manufacturer
Starter day 0–15	Crude protein 17.5, calcium 0.8, phosphor 0.64, lysine 0.96, methionine 0.47	Opfmeel F1 101961, De Heus voeders BV
Grower day 15–32	Crude protein 20.3, calcium 0.92, phosphor 0.62, lysine 1.32, methionine 0.63	94017 V/K ST/GR GD, ABZ Diervoeding
Finisher day 32–44	Crude protein 13.9, calcium 3.3, phosphor 0.43, lysine 0.66, methionine 0.32	Foktoom SLF F2 kruimel 101782, De Heus voeders BV

2.7. Behavioural tests

2.7.1. Tonic immobility

The tonic immobility (TI) test was conducted on day 3 and 4 between 9:00 and 17:00 by one experimenter. Four birds of each treatment were used to practice the procedure, thus a total of N = 82 (N = 41 per experimental treatment) were included in the analysis. Chicks were selected for testing by alternating between the pens and picking an individual, alternating between individuals that approached readily and individuals that maintained more distance. The TI test took place in an adjacent room. The chick was placed on its back on a cradle and light pressure was applied to the chest with one hand for 10 s. If the duration of TI was less than 5 s, it was noted as a failed induction attempt and the chick was restrained again, for a maximum of 4 times in total. If the bird did not turn upright within 5 min, the maximum score of 300 s was given to limit the discomfort of the chickens (Mignon-Grasteau and Minvielle, 2003). The number of induction attempts and the latency to righten (standing on both legs) were recorded for chicks in which TI was successfully induced (N = 76).

2.7.2. Open field

The open field test was conducted twice for each chick; on days 9–11 (OF1), and on days 37–39 (OF2) between 09:00 and 17:00. The open field arena (1 ×1 m) was placed in a room adjacent to the pens. The floor of the arena was covered with the same bedding as used in the pens. A digital video camera (DVC) was placed in the middle above the arena to make video recordings of all trials. The system to operate the DVC was located outside the open field room to minimise distraction of the chickens. The recordings were scored by two observers blind to the treatment of the focal chick with The Observer® XT (version 12; Noldus, Wageningen, the Netherlands), using the continual focal sampling method to score the behaviours in the ethogram in Table 3. Next to behaviour, ambulation (number of lines crossed) was scored during OF1 by dividing the arena in 9 squares during the video analysis.

Both observers scored three of the recordings twice, and three recordings were scored by both observers to be able to check intra- and interrater reliability. Cohen's Kappa for intra-rater reliability was scored at 0.69 for CD and 0.71 for MJ, and 0.68 for inter-rater reliability. These levels of Cohen's Kappa are considered moderate or substantial, depending on the interpretation (McHugh, 2012), and were considered to be sufficient.

Depending on the behaviour, the latency, duration or frequency is used in later analysis, see Table 4 below.

Table 3
Ethogram for the open field test, adapted from (Campler et al., 2009; Daigle and Siegford, 2014; Fraess et al., 2016).

Locomotive	Walk	Chicken is walking more than 3 steps in succession with head up or when walking chicken has not been standing, drinking, feeding, or foraging in litter for the previous 5 s
	Escape attempt	Jumping into the air and beating wings to extend fall while moving away from original location.
Immobile	Stand/sit	Immobile in standing/sitting position (sternum resting on ground).
	Stand/sit alert	Immobile in a standing or sitting posture with eyes open and an alert body stance
Oral behaviours	Forage	Chicken pecks at substrate on ground while standing or stepping forward with head below rump level. Starts when chicken makes > 3 successive pecks at substrate, or when foraging chicken has not been standing or walking with head up, or feeding, for the previous 5 s
	Explore peck	Pecking at arena or other objects
Maintenance	Preen	Uses beak to trim and arrange feathers
	Feather ruffle	Erects feathers away from skin, puffing and ruffles up, and shakes body.
	Wing Flap	Beating wings while body is kept upright.
	Leg Stretching	Stretching one leg often together with the wing of the same side, but also may be stretched alone while sitting or standing.
	Defecation	Defecates during the test

Table 4
Overview of behavioural variables measured during the OF test.

Latency	First step, vocalisation
Duration	Walking, stand/sit, stand/sit alert, forage
Frequency	Defecations, escape attempts, explore pecks, preen, feather ruffle, wing flap, leg stretching.

2.8. Feather analysis: corticosterone and quality

At 36 days of age, primary feather 2 was collected from the left wing of each chicken. The feather was cut at the base and each feather was stored in a labelled envelope in the dark at room temperature until used for the CORT analysis. The protocol for CORT extraction was based on (Bortolotti et al., 2008).

Feathers were washed with 100% methanol (1.06009.2500, Merck, Germany) and left on a piece of paper in a fume hood until completely dry. The tip and calamus (until the first downy barbs) were removed and the feather was measured and weighed to the nearest 0.0001 g. The rachis was removed and the vanes were cut and weighed. After adding steal beads and 5 mL 100% methanol the tubes were placed on a shaker at 8000 rpm for 10 min. The tubes were then wrapped with aluminium foil to prevent light degradation of CORT and placed in a roller mixer overnight. The next morning, the tubes were centrifuged twice and 1.0 mL of the supernatant evaporated in a Speed Vac Concentrator (CentriVap Concentrator Labconco) for 2 h at 42 ºC. The dried extracts were dissolved in 300 µL ELISA buffer provided with the commercial assay kit we used to determine CORT concentrations (Cayman, kit 501320, batch 0559915, Ann Arbor, MI, USA). The results from the assay were corrected for feather length and mass.

To assess feather quality, photographs were taken of the collected primary feather 2 prior to processing for CORT analysis. The feather was scored on two characteristics: feather damage and presence of tip. Feather damage was scored from 0 to 2, with 0: no damage, 1: few fault bars, 2: fault bars and feather breakage (based on Møller et al., 2009). In contrast to Møller et al. (2009) we chose to score the general appearance of the feather instead of counting the fault bars.

2.9. Faecal corticosterone metabolites

During both open field tests, after each individual trial, droppings were counted and collected in plastic bags for analysis of faecal CORT metabolites (FCM). Samples were kept on ice for less than 4 h, then frozen at − 80 °C until analysis in April 2021. Samples were thawed, straw and other debris was removed, then homogenised and weighed to the nearest 0.1 mg. Afterwards, samples were dried in a stove at 70 °C for 48 h. The dry samples were weighed again to calculate the wet weight/dry weight ratio. The dried samples were homogenised to powder using a tissue lyser and one steal ball (50 Hz, 5 min). From each sample, 0.05 g were transferred to a clean 2 mL tube and 1.5 mL ethanol 60% was added. Samples were then vortexed for 30 min (Multivortex, setting 8, 14,000 rpm), then centrifuged at 14,000 rpm for 20 min at room temperature in a tabletop centrifuge. The supernatant was transferred to a clean tube and evaporated in a Speed Vac Concentrator (CentriVap Concentrator Labconco) at 42 °C 90 min. To determine FCM concentrations, samples were measured using a commercial radioimmunoassay kit, following the protocol of the supplier but using half the indicated amounts (MP Biomedical ICN Corticosterone Double Antibody 125I RIA Kit, 0712010-CF). Steroid diluent, provided in the assay kit, was added to each tube to dissolve the residue. The kits were validated for measuring FCM in chicken samples by measuring serial dilutions and adding a known amount of CORT standard solution.

2.10. Statistical analysis

The experimenters were blind with respect to the transportation treatment; unblinding took place after finishing all behavioural tests and video analyses.

Data were analysed using the statistics software programme RStudio (2021.09.1 Build 372) (R Foundation for Statistical Computing, 2021) and following packages: 'readxl', 'lme4′, 'ggplot2′, 'tidyr', 'dplyr', 'psych', and 'MASS' (Venables and Ripley, 2002; Wickham, 2009, 2021; Bates et al., 2015; Revelle, 2021; Wickham and Bryan, 2022; Wickham et al., 2022). A significant effect was accepted for $p < 0.05$. Figures were prepared in using IBM SPSS Statistics version 27. Error bars in figures represent the standard error of the mean (SEM), unless indicated otherwise. Raw data and model estimations plus confidence intervals are included in Supplementary File 1.

Models were fit with treatment (ST, LT), sex (F, M) and their interaction set as fixed effects. Pen (1−4) was added as block factor to all models and was retained in the final models. Reference categories for models were set to sex: female and treatment: short transport. Residual plots were inspected to assess the model fit. Backward model selection was done based on comparison of the AIC (smaller is better). Treatment was always retained in the final model.

Estimates (differences between group means) with the 95% confidence interval (CI) are reported for linear models and an effect was considered significant ($p < 0.05$) if the 95% CI did not include zero. In the case of log transformed data and models with a link function (Poisson, negative binomial, quasi Poisson) the estimate represents the ratio between group means (LT vs. ST) and the 95% CI not including 1 (i.e. equal means) was considered significant ((OF behaviours, Faecal CORT metabolites (FCM)). Continuous data (egg characteristics, tarsus length day 44, behaviours during open field tests (OF1 and OF2), feather CORT and FCM) were analysed with general linear models with a Gaussian distribution. Body mass at day 1 was analysed by adding egg mass as predictor to the model. Tarsus length at day 44 was analysed by adding tarsus length at day 1 as predictor to the model. Behaviours shown by less than five animals per category were not analysed (preening, feather ruffle, wing flap and leg stretching, and foraging during OF1). CORT in feathers was analysed based on two units, amount of CORT (in pg) per mg feather and amount of CORT (in pg) per cm feather.

Scores (health scores, feather damage, TI induction attempts) were

converted to 0/1 (no damage (score 0) vs damage (score >0)) due to the low sample sizes in higher score categories. The scores were analysed with a Chi-square test for proportions. Count data was analyses by fitting a Poisson link distribution (OF: explorative pecks, escape attempts, defecations), or a quasi-Poisson distribution (TI induction attempts).

Latency to righten in the tonic immobility (TI) test was visualised with a Kaplan-Meier table and analysed with a cox proportional hazard analysis.

Repeated measures (heart rate, body mass) were analysed with a linear mixed model (library lme4), with timepoint of measurement as additional fixed effect and chick ID set as random effect. Backward model selection was done based on maximum likelihood estimation and the final model was estimated with REML. The eight heart rate measurements per time point were averaged per individual egg and the average was used as dependant variable. Covariance structure for the heart rate model was set as unstructured. To investigate treatment effects on body mass gain, a corAR1 correlation structure was set as covariance structure and a power variance function was added to cope with the increasing variability of the data due to increasing age and differences between males and females.

3. Results

3.1. Sexes

All birds were sexed based on the feather PCR products and secondary sexual character (see Table 1 for distribution of the sexes across pens and treatments).

3.2. Embryonic heart rate measurement

Mean heart rate significantly increased with measurement timepoints ($p < 0.001$) from baseline (HR0, mean 134.1 bpm, 95%CI = [125.9, 142.2]) to measurement timepoints HR1 (+13.7 bpm, 95% CI = [7.4, 20.0]), HR3 (+29.0 bpm, 95% CI = [21.0, 37.0]) and HR5 (+26.8 bpm, 95% CI = [18.9, 34.8]); Fig. 1. Between HR3 and HR5, no further significant change in heart rate was observed (HR5 vs. HR3, −2.2 bpm, 95% CI = [−10.9, 6.6]).

The interaction between sex and measurement time point was not significant ($p = 0.79$), and heart rate did not vary between the sexes (males: −4.6 bpm, 95%CI = [−13.4, 4.2], $p = 0.29$).

3.3. Body mass, tarsus and health scores

There was no statistical difference in mass, length, or width of fresh eggs between treatment groups (ST, LT) or sexes (Data found in Supplementary File).

Body mass at day 1 (mean 22.8 g, 95% CI = [13.8, 31.7]) did not significantly differ between treatments (mean LT −0.4 g, 95% CI = [−1.6, 0.8]). Body mass gain was not affected by treatment (mean LT −0.6 g, 95% CI = [−2.0, 0.7], $p = 0.35$), but males grew heavier over time compared to females (time * sex, $p < 0.001$, Fig. 2). On day 43, at the end of the experimental period, males were on average 153.4 g (95% CI = [61.9, 245.0]) heavier than females; Fig. 2).

Treatment did not affect tarsus length (LT mean −0.03 mm, 95% CI = [−0.22,0.16], $p = 0.72$) and neither did the interaction of treatment and sex ($p = 0.91$). On day 44, females had significantly shorter tarsi (mean 6.62 mm, 95% CI = [6.38, 6.86]) compared to males (mean +0.65 mm, 95% CI = [0.46, 0.84]).

All birds scored 0 for breast discoloration/blisters and hock burns; one ST birds scored 1 for skin lesions. Scores for plumage cleanliness and foot pad lesions (left and right) did not statistically differ between treatments (Supplementary Data File).

3.4. Behavioural tests

3.4.1. Tonic immobility

Two ST (out of 41, 4.9%) and 4 LT (out of 41, 9.8%) birds did not enter TI (treatment 95% CI = [−0.09, 0.19], $p = 0.67$). The mean number of induction attempts (1−4) did not differ between treatment groups (meanLT/meanST 1.17, 95%CI = [0.67, 2.08], $p = 0.58$).

The latencies to righten did not differ between the sexes (males vs females, hazard ratio 0.86, 95% CI = [0.5, 1.4], $p = 0.53$) or the treatments (LT vs ST, hazard ratio 0.98, 95% CI = [0.6, 1.6], $p = 0.94$, Fig. 3).

3.4.2. Open field

Treatment did not affect most of the behaviours measured during the open field tests (Table 5).

During the OF2, however, LT birds performed more explorative pecks (Table 5), and ST males performed less escape attempts compared to ST females (ST males vs ST females 0.48, 95% CI =[0.23, 0.96], $p = 0.037$), while LT males and females did not differ (LT males vs LT females, 1.01, 95%CI = [0.52, 1.98]). Overall, males spent less time walking during the OF2 (Table 5).

3.5. Feather corticosterone and quality

There was no interaction between treatment and sex (pg/cm: $p = 0.55$; pg/mg: $p = 0.4$, ST: N = 16 female, 18 male; LT: 17 female, 18

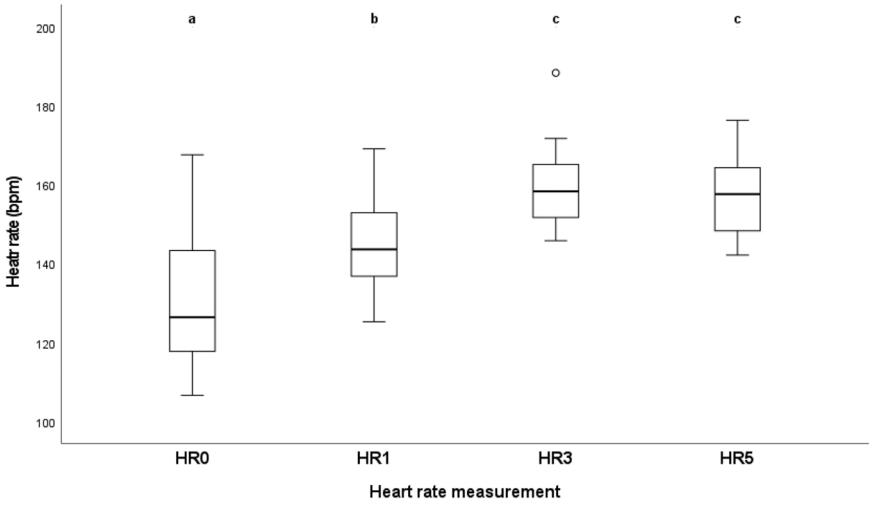

Fig. 1. Embryonic heart rate (beats per minute) measured before transport (HR0), after 1 (HR1), 3 (HR3) and 5 (HR5) trips, respectively. Box plot centre lines show the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, the outlier is represented by a circle (upper quartile plus 1.5 times IQR). Sample size (males/females): HR0 = 23 (16 */7), HR1 = 24 (17/7), HR3 = 12 (9/3), HR5 = 12 (9/3). * Measurement of one egg failed, due to machine error. Superscript letters indicate statistical differences.

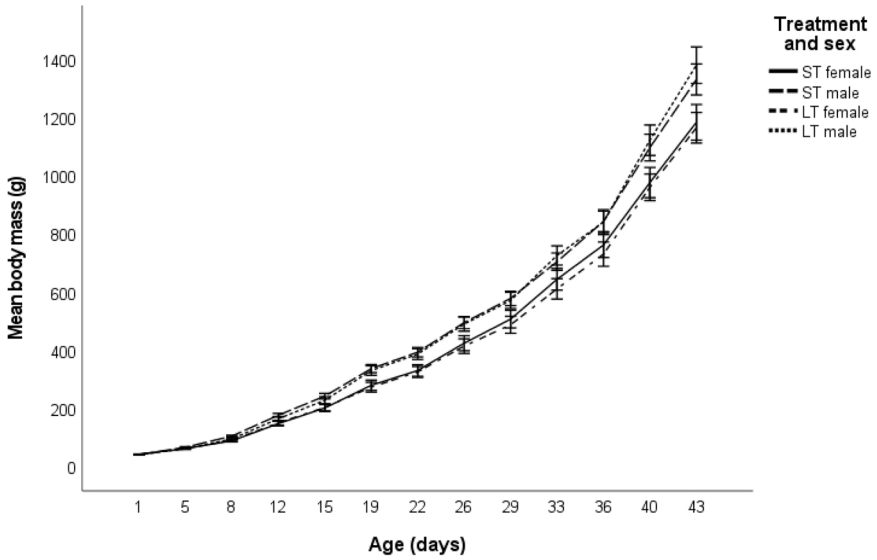

Fig. 2. Mean body mass (SEM) over the course of six weeks (two measurements per week), starting with mass at hatch. ST = short transport (N = 20 female, 25 male), LT = long transport (N = 22 female, 22 male).

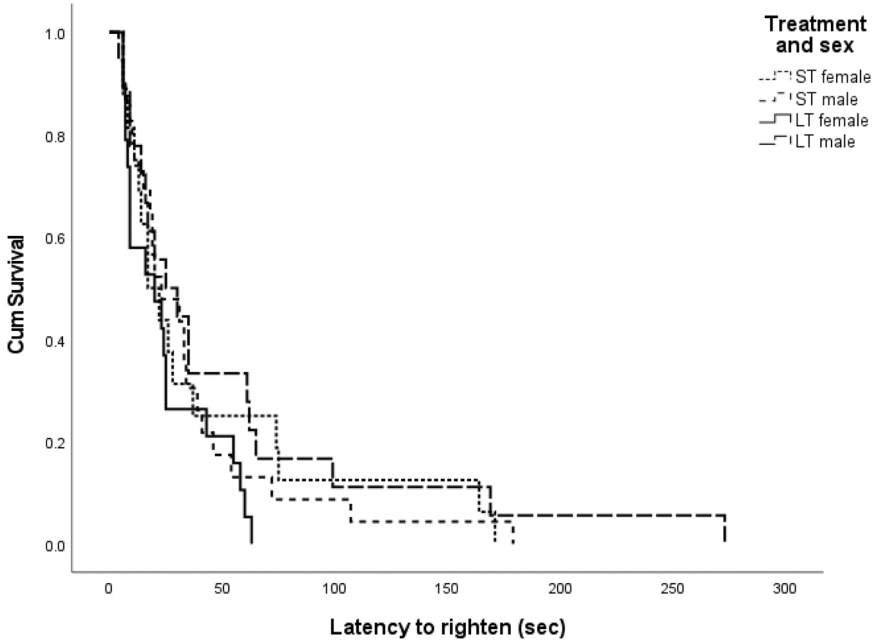

Fig. 3. Survival graph of the latencies to righten during the tonic immobility (TI) test. ST = short transport (N = 16 female, 23 male), LT long transport (N = 19 female, 18 male).

male) on CORT concentration in feathers. The main effect of sex was not significant (males pg/cm: −0.17, 95% CI = [−0.60, 0.25], p = 0.43; pg/mg: −0.35, 95% CI = [−0.72, 0.03], p = 0.08). Also the main effect of treatment was not significant (raw means pg/cm: ST 3.38 (0.97 SD), LT 3.27 (0.89 SD); treatment: − 0.13, 95% CI = [− 0.55, 0.29], p = 0.65; raw means pg/mg: ST 2.56 (0.88 SD), LT 2.48, (0.88 SD); treatment: − 0.11, 95% CI = [− 0.49, 0.27]), p = 0.71).

Feather damage did not differ between treatments (p = 0.36, see Table in Supplementary Data File). Feather mass (0.0002 g, 95% CI: [−0.0039, 0.0043]) and length (0.003 mm, 95% CI[−0.377, 0.383] did not differ between treatments.

3.6. Faecal corticosterone metabolites

Not all birds defecated during the OF tests. During the OF 1, droppings were collected from 17 ST females, 24 ST males, 19 LT females, and 20 LT males for FCM analysis. During the OF 2, droppings were collected from 14 ST females, 20 ST males, 16 LT females, and 18 LT males for FCM analysis.

The interaction between treatment and sex significantly predicted log FCM concentrations during OF1 (p = 0.008, Fig. 4). ST females (64.18 ng/g, 95% CI =[50.36, 81.78]) had 30% higher FCM concentrations compared to ST males (0.70, 95% CI = [0.55, 0.89], p = 0.005), while LT females and LT males did not differ (1.11, 95% CI = [0.86, 1.42], p = 0.43).

For the log FCM concentrations measured from samples collected

Table 5
Effects of treatment (ST reference category) on mean behaviour during the first open field (OF1) and second open field (OF2) test.

Behaviour	Session	Transformation	N	N ST	Raw mean ST	ST SD	N LT	Raw mean LT	LT SD	Treatment (estimate)	95% CI	p value
Latency to first step (sec)	OF1	log	86	45	6.01	7.41	41	8.97	11.21	1.34	[0.79, 2.40]	0.31
	OF2	log	89	45	15.22	21.28	44	17.18	13.35	1.806	[0.996, 3.273]	0.055
Walk duration (sec)	OF1	none	86	45	154.70	67.31	41	169.02	75.00	13.93	[− 16.48, 44.34]	0.37
	OF2	log	89	45	39.40	35.24	44	48.26	34.48	1.08	[0.75, 1.56]	0.66
sex effect (males vs females)	OF2	log								sex (0.56)	[0.39, 0.81]	0.003
Stand/sit duration (sec)	OF1	none (robust linear regression)	85	44	96.75	67.88	41	89.70	68.99	-12.41	[− 40.06, 15.24]	1.00
	OF2	none	89	43	176.72	118.74	43	142.49	93.26	-31.98	[− 77.32, 13.36]	0.17
Stand/sit alert (sec)	OF1	log	86	45	223.08	77.48	41	212.55	90.48	0.92	[0.77, 1.11]	0.41
	OF2	log	89	45	206.48	106.56	44	237.10	95.50	1.20	[0.98, 1.47]	0.08
Forage duration (sec)	OF1	not analysed	8	6	4.85	4.09	2	5.16	3.67			
	OF2	log	52	25	91.10	98.33	27	69.99	62.60	0.85	[0.40, 1.82]	0.68
Explore peck (count)	OF1	neg binomial	45	25	10.96	20.33	20	15.40	28.61	1.32	[0.56, 3.07]	0.47
	OF2	neg binomial	27	13	2.77	2.24	14	6.07	5.97	**3.20**	**[1.57,6.80]**	**0.001**
Escape attempt (count)	OF1	log	82	42	7.33	5.01	40	9.88	11.72	1.28	[0.93, 1.76]	0.13
	OF2	neg binomial	42	21	2.76	2.95	21	3.29	2.67	**treatment * sex**		**0.045**
Defecations (count)	OF1	quasi Poisson	83	45	2.31	1.31	42	2.02	0.98	0.88	[0.70, 1.09]	0.25
	OF2	Poisson	89	45	1.53	1.14	44	1.86	1.41	1.22	[0.88, 1.68]	0.23
Latency to vocalise (sec)	OF1	log	86	45	11.73	30.66	42	7.14	8.10	0.91	[0.64, 1.30]	0.61
	OF2	log	89	45	20.60	41.29	44	13.16	10.74	0.95	[0.61, 1.48]	0.81
Ambulation (nr. of lines crossed)	OF1	log	86	45	72.51	39.83	41	76.05	40.73	1.03	[0.81, 1.31]	0.82
	OF2		not measured				not measured					

Unless the effect of sex was significant, only the main effect of transport treatment is reported (estimates and 95% CI). Estimates (differences between group means) with the 95% confidence interval (CI) are reported. An effect is considered significant ($p < 0.05$) if the 95% CI does not include zero, unless raw data was log transformed for analysis in which case the estimates represent the ratio between group means and the 95% CI not including 1 (i.e. equal means) is considered significant. N refers to the number of birds showing the behaviour (during OF1, 86 animals were included in the analyses, three were used for testing the set-up, during OF2, all 89 birds were tested).

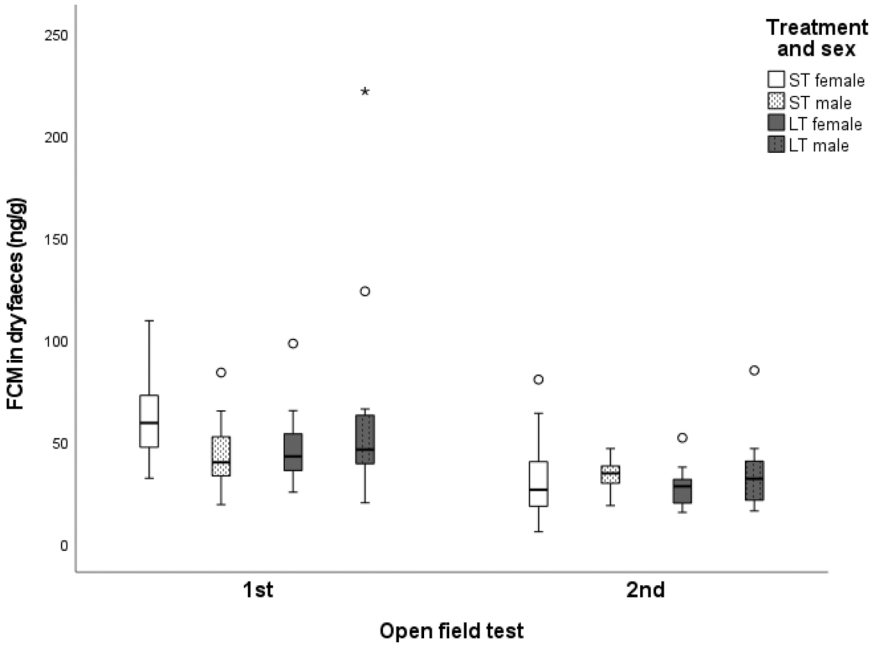

Fig. 4. Faecal CORT metabolite concentrations (FCM, in ng per g dried faeces) in droppings collected during the first open field test (OF1, left) and the second open field test (OF2, right) for short transport (ST) and long transport (LT) female and male birds. Box plot centre lines show the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles, outliers are represented by circles (upper quartile plus 1.5 times IQR), extreme outliers by an asterisk (upper quartile plus 3 times IQR). Sample size OF1 (ST: N = 17 female, 24 male, LT: N = 19 female, 20 male), OF2 (ST: N = 14 female, 20 male, LT: N = 16 female, 17 male).

during OF2 (Fig. 4), the interaction between treatment and sex was not significant ($p = 0.56$). The FCM concentration did not significantly differ between sexes (males 1.17, 95% CI = [0.95, 1.43]) or treatment groups (LT 0.95, 95%CI = [0.77, 1.16]).

4. Discussion

The present study investigated the behavioural and physiological effects of *in ovo* transportation of broiler chickens on E18. In line with our major hypothesis, we found that the embryonic heart rate increased with increased transportation time. We did not, however, observe long term effects of transportation on chick growth, behaviour or measures of CORT in feathers or faeces in the present study.

4.1. Transportation increases embryonic heart rate

We hypothesised that the longer the embryo was transported, the more the heart rate would increase in a response to the stressors brought about by transportation (e.g temperature changes, vibrations and accelerations). For the majority, this expectation holds true. Heart rate increased significantly after 1 and 3 trips compared to before transportation. We also observed an increase in heart rate between 1 and 3 trips. However, it appears that the increase in heart rate plateaus after 3 trips, as no significant difference is found between the heart rate measured after 3 and 5 trips. There are few studies, to our knowledge, that measure embryonic basal heart rate utilising the Egg Buddy® (Lierz et al., 2006; Bertin et al., 2015, 2018). Therefore, it is difficult to compare the pattern in heart rate observed in the present research to results from other studies on embryonic heart rate of broiler chickens. However, our results clearly demonstrate the importance of taking exposure to acute stressor *in ovo* into account given the clear responses of the embryos to transportation.

4.2. Body mass and tarsus length are not affected by length of embryonic transport

Body mass was expected to be lower for the LT group at hatch, and this difference was expected to remain present for the other weight measurements taken in the weeks following, as a long-term consequence of the early exposure to longer transportation. The expected differences were not found for any of the weighing moments. There was a sex difference found in chick body mass between day 8 and 29, with males significantly heavier than females during this period. A similar pattern was found for tarsus measurements, as no effect of treatment was found, but on day 44 the tarsus of males was significantly longer than that of females.

The absence of a difference in hatching weight is in line with results found by Janczak et al. (2006) following exposure of layer chicken embryos to CORT very early in embryogenesis (day E0). However, in contrast to the present study, chicks treated with CORT *in ovo* showed lower body mass as chicks at 1 and 4 weeks of age. A similar result was found by Eriksen et al. (2003), who reported that after 3 and 11 weeks, broiler chickens injected with CORT *in ovo* had a significantly lower body weight. Effects of CORT injection *in ovo* on body weight are not always congruent; a follow-up study by (Janczak et al., 2007) that prenatally administered CORT in a lower dose did not find any differences between treatment for the body weight measurements in week 3. In the present study, we presume that exposure to transport stress in embryos, which leads to an elevation in heart rate, will also lead to CORT release; this may be at levels low enough that body weight is not affected. Alternatively, transportation stress at day E18, considerably later in embryogenesis, may not have as strong an effect as activation of the HPA-axis during very early development, such as in the studies cited above. An effect of post-hatch transportation duration on broiler chick weight is also observed in a study by (Khosravinia, 2015), which shows that weight loss of the one-day old chicks continues to occur linearly per 100 km of journey; this is however a quite different situation compared to *in ovo*, where lack of feed and water during transportation of hatched chicks may also play a role which is not expected during *in ovo* transport.

As is the case for the body mass results, there is no detectable difference in tarsus length between the treatments. There are few studies that take tarsus length into consideration following embryonic manipulations. A study on barn swallows (*Hirundo rustica*) by (Saino et al., 2006) showed that chicks from CORT-injected eggs had shorter tarsi. For future studies it might be interesting to look at the asymmetry of the tarsus in addition to merely tarsus length. Asymmetry has been indicated to be increased in chicks prenatally exposed to CORT (Eriksen et al., 2003); increased prenatal CORT levels due to transportation are also expected in the present study, thus it might be interesting to consider this parameter.

4.3. Behaviour is not affected by length of embryonic transport

Our behavioural results were contrary to our original hypothesis that individuals from the LT group would show more fearful and less explorative behaviour during the tonic immobility and open field tests, as a long-term result of the prenatal stress due to transportation. None of the observed behavioural parameters showed any significant differences between the ST and LT group.

We predicted a difference in fear-related and exploratory behaviour following short- or long transportation based research that shows behavioural differences as a result of early-life stress (Forkman et al., 2007; Elfwing et al., 2015). The relationship with *in ovo* stress and fear is, however, complex. For instance, prenatal exposure of broilers to CORT showed effects of the treatment on tonic immobility in interaction with postnatal handling (Janczak et al., 2007). The same goes for the open field tests in the research by (Henriksen et al., 2013), that did not find any significant differences between the CORT- and the control group in laying hen chicks treated *in ovo*.

During the second open field test, a trend was detected in the total duration the chickens were standing or sitting alertly. The LT group showed a tendency to adopt an alert position for a longer total period of time than the ST group, which might be an indication that the early stress from transportation has had a long-term effect. Research on the red junglefowl (ancestor) and White Leghorn chicken (domesticated, less fearful) showed that the more fearful red junglefowl also spends a longer period standing/sitting alertly during an OF test (Campler et al., 2009). Thus the trend found in the current research might be a sign of increased fearfulness. However, considering that it is the only trend that has been detected and no significant differences were found, if there are differences in fearfulness due to transportation, they are likely to be small differences or variable between individuals.

4.4. Feather corticosterone, faecal corticosterone metabolites and feather quality

In contrast to our hypotheses, feather CORT levels were not found to differ between the LT group and the ST group. Similarly, no differences found in feather quality between the treatments. We also did not observe any differences between the experimental treatment groups in CORT metabolite levels in faecal matter.

Research has shown that CORT deposition in feathers can provide a representation of stress during feather growth in birds (Bortolotti et al., 2008; Jenni-Eiermann et al., 2015) and that exposing layer hens to CORT through drinking water, increases CORT as measured in feathers (Bartels et al., 2021). High stocking density during rearing also increased CORT levels measured in feathers in layer hen pullets (von Eugen et al., 2019). However, research on environmental enrichment has shown that even if stress-related behavioural differences are present, this may not be visible in feather CORT levels (Fairhurst et al., 2011). In addition, differences in rearing conditions do not always affect CORT feather levels later in life (Nordquist et al., 2020). This indicates that a

very robust stimulus may be necessary in order to find differences in CORT levels in feathers.

As for the feather quality, past research has shown that CORT-implanted feral pigeons (*Columba livia domestica*) show altered feather growth, with a change in structure and colour (Jenni-Eiermann et al., 2015). Fault bars in feathers are also more frequent under stressful circumstances (Møller et al., 2009). When looking at the absolute number of feather damage scores (see Section 3.5), there is a difference visible, with the LT group showing more high feather damage scores. However, this difference is not statistically significant. As the sample size of this study is relatively small, in a study with a larger sample size it might be possible to detect such an effect.

4.5. Limitations

An important limitation of the present study is that the eggs from both groups were transported, which means that there was no control group that had not been transported at all. This was done for practical reasons: the eggs needed to be transported to the experimental facilities in order to all be hatched from the same hatchers. Even though the LT group was transported five times longer than the ST group, we cannot rule out that the short transport may have resulted in long-term effects on behaviour and physiology. We demonstrated that after the short transportation, the embryonic heart rate was already significantly increased as compared to prior to transportation. This may have resulted in ceiling levels of stress-induced changes, which could then mask long-term effects of transportation stress.

Another difference between the present study and on-farm hatch in practice is that the eggs were also placed in a setter at E18 to further incubate, then transferred to pens after hatching. In on-farm hatch, the eggs would be placed in the barn to incubate and hatch, rather than a setter. We cannot rule out that hatching in a hatcher and then moving the chicks to a pen provides an additional stressor to the chicks. This also could potentially overshadow effects of *in ovo* transportation.

Furthermore, the housing conditions for the broiler chicks in the present study differed from those in the conventional broiler farming in terms of stocking density group size and enrichment, all of which have been shown to affect physical (Thomas et al., 2004; Estevez et al., 1997), physiological (Beloor et al., 2010), and behavioural (Fairhurst et al., 2011) responses. Consequently, it is possible that the set-up of the present experiment with comparatively small groups of animals, low stock densities, and much enrichment alleviated or even reversed the stress experienced during transportation.

4.6. Future research and recommendations

The present study is, to our knowledge, the first to investigate the long-term behavioural and physiological effects of transport of broiler chicken *in ovo* on E18. Because of the novelty of the research, there are many avenues to continue and expand the current research in addition to those mentioned in previous sections.

First, it could be of value to research which aspect of transport is most stressful (e.g. noise, temperature changes, vibrations or acceleration); *in ovo* chronic noise exposure, for instance, has been demonstrated to affect later behaviour in young quail (Mezrai et al., 2022). Temperature, including local differences in temperature, may also be a stressor. It is important to realise that day-old chicks often regulate their temperature behaviourally, moving to warm areas when cold and vice versa. This is obviously not a possibility *in ovo*, thus temperature fluctuations could potentially be more stressful to chicks *in ovo*.

Second, more parameters could be tested if the present research were to be repeated. For instance, more behavioural tests could be carried out, as prenatal CORT exposure has been shown to affect memory in birds (Sui et al., 1997; Rodricks, 2006) and decrease dominance in male birds (Lay and Wilson, 2002). Finally, it would be of interest to examine the acute responses to transportation more closely, by for instance also measuring acute CORT responses and/or other physiological measures related to stress physiology.

Based on the results of the present research, we would recommend limiting chicken transport as much as possible, including *in ovo*, and working to optimise transport of both chicks and eggs. Nonetheless, when comparing the results from the present study with research on transportation of one-day old chicks (Mitchell, 2009; Khosravinia, 2015; Vieira et al., 2019), there appear to be more adverse effects of transportation at the age of one day. In combination with some promising results of the on-farm hatching system, the introduction of on-farm hatch may be a positive development for the broiler industry, including the welfare of the broiler chickens.

5. Conclusion

The present study was designed to test whether transportation of eggs at E18 results in significant differences in the behaviour and physiology both *in ovo* and post-hatch. The early exposure to longer transport resulted in a significant increase in heart rate, hence there seems to be a short-term effect on the physiology of the embryo. No long-term effects were detected on body mass, tarsus length, fearful behaviour during a tonic immobility test and open field tests, nor CORT levels in feathers or faeces. Our study indicates that transportation *in ovo* does not appear to have long-term effects on behaviour and physiology, but does have an acute effect on chick embryos. Optimal *in ovo* transportation should be taken into account in poultry farming to minimise exposure to stress.

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CRediT authorship contribution statement

Rebecca E. Nordquist: Conceptualization, Writing – review & editing. **J.C.M. Vernooij:** Formal analysis, Data curation. **C.L. Dull:** Formal analysis, Investigation, Data curation, Writing – original draft. **A. Pascual:** Investigation. **G. van der Linde:** Conceptualization, Methodology, Resources, Writing – review & editing. **Vivian C. Goerlich:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

GL was employed by Heering Transport, a company that manufactures trucks for transportation of eggs and poultry, at the time the experiments were conducted. Heering contributed in-kind by providing the transportation of the eggs for these experiments; no financial contribution from Heering was involved. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2022.105789.

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