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Women in animal behavior and welfare: 2021

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Women in animal behavior and welfare: 2021

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Editorial: Women in animal behavior and welfare: 2021

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Editorial on the Research Topic
Women in animal behavior and welfare: 2021

The work of female scientists often does not receive the attention it deserves. To recognize and promote the achievements of women in animal behavior and welfare science, this Research Topic aims to highlight the scientific contributions of women researchers in this area. In animal behavior and welfare science, as a relatively young field, which gained increasing scientific importance just over the last two decades, the proportion of female researchers is at a high level already. However, no documented numbers regarding gender balance exist.

With this Research Topic, we honor the merits of female scientists in animal behavior and welfare science, a field of great public interest that is increasing even still. The subject of this scientific field is the mental and physical state of an animal while interacting with its living environment (i.e., health, care, stress, feeding and supplemental feeding, learning and stimuli enhancement). Traditional methods such as animal behavior assessment via direct observations have been technically improved over the last decade by up-to-date techniques such as advanced video recordings and automatic analyses of behavioral traits and complete ethograms. Another groundbreaking aspect is the new multi-omics profiling approach, the existence of molecular mechanisms allowing genotype-environment interactions, the so-called epigenetic mechanisms. According to this theory, the living environment selects which gene has to be turned on and which one to be turned off. Transferred to animal welfare, this means that positive environmental stimuli guarantee animal welfare, while negative stimuli predispose to the onset of various diseases (1). The epigenetic mechanism as a final effect has an impact on the animal phenotype, and, therefore, on its welfare and the development of behavior (2–4).

The variety of different scientific methodologies and approaches to evaluate and improve animal welfare in different species is presented in the contributions to this Research Topic. The papers do not only consider the most relevant livestock species but deal with other animals kept for human use, for instance as laboratory animals such as zebrafish, as in the research carried out by Leyden et al. In their study, the impact of tricaine, the most commonly used chemical anesthetic in zebrafish research, on different physiological parameters is thoroughly evaluated and compared to gradual cooling. New insights were generated, but the results also clearly show the need for further research regarding the potential of appropriate alternative anesthetic agents for the sake of zebrafish welfare.

Staying with aquatic species, but concentrating on invertebrates, Wahltinez et al. provide a comprehensive overview of this hitherto neglected topic in their perspective article. Aquatic invertebrates, such as cephalopod mollusks and decapod crustaceans, can suffer stress and feel pain, too. The authors encourage the protection of aquatic invertebrate welfare and provide practical recommendations using anesthesia, analgesia, and euthanasia in addition to non-invasive handling methods in aquaculture and fisheries. With this important contribution, the authors advocate further research in this underrepresented but important field of animal welfare.

Changing to poultry, one major welfare issue in laying hens is feather pecking, often followed by cannibalism. One prevention measure is the provision of an adapted feeding regime with supplements. In their study, Mindus et al. analyze the impact of dietary supplementation with *Lactobacillus rhamnosus* JB-1 probiotic bacteria against stress-induced severe feather pecking damage. Based on their results, the authors suggest that this probiotic strain may have beneficial effects on the avian immune response and the prevention of feather pecking and plumage damage, thus increasing animal health and welfare.

Junghans et al. provide an exploratory study on the evaluation of fattening and slaughtering of broiler chickens by multivariate analyses, considering different factors comprehensively. Several factors were identified that can affect the mortality of broilers during the rearing period, their slaughter weight, and the causes of condemnation recorded at the processing plant. With these new insights, the authors show the potential of minimizing the use of antibiotics on farms where animal welfare is ensured.

Comprehensive statistical analyses on the base of a large data set were also the basis of the research presented by Dachrodt et al. They not only give a detailed overview of the status quo of colostrum, feeding, and housing practices of pre-weaned dairy calves in German dairy farms but also developed a benchmark system to evaluate calf health on farms and to identify potential problem areas. For all persons involved in calf management, such as farmers, herd managers, veterinarians, and other advisors, this tool is beneficial to assess on-farm calf health and thus brings this topic, for the benefit of calves' welfare, into focus.

On a more experimental level, Stenfelt et al. explored whether dairy cows have the cognitive abilities to learn new behavior *via* social learning. In their experiments, they showed that cows did not utilize social learning mechanisms when solving a spatial detour task. The knowledge of social learning in farm animals is very limited, and with these new insights, the authors provide essential new information and open the space for further research questions concerning the cognitive abilities of cattle.

Other milk-producing species, more precisely sheep and goats, are present in this Research Topic, too. The study carried out by Berthel et al. describes the preference of non-lactating dairy sheep and goats for a diet containing a monocomponent vs. a mixed ration of the same components and similar nutritional value. This new aspect can be used in creating adapted diets, considering ruminants' natural behavior of selective feeding, and improving their wellbeing in that way.

To close the circle of animal species, Carroll et al. present a study evaluating the prevalence of adoption and relinquishment of dogs and cats during the COVID-19 pandemic. They identified risk factors for relinquishment and put, with this innovative study, the topic into focus. Especially for information on prevention and interventions aiming at the reduction of companion animal relinquishment, these findings are of utmost importance.

Finally, in the review article by Krebs et al., the influences of space, time, and context on patterns of anticipatory behaviors in animals under human care are discussed intensively. Unidentified anticipation can alter conclusions regarding animal behavior or welfare under certain circumstances, and the authors explain for instance, how animals are driven to anticipatory behavioral models by reward desire. With this work, valuable advice is given on how such impairments in animal welfare research can be identified and taken into account.

Concluding, the body of research included in this Research Topic impressively shows the various contributions female scientists bring to the field of animal behavior and welfare research. By providing science-based results to increase the knowledge of the effects of the living environment on animal welfare and behavior, useful, practical approaches to improve the welfare of a variety of species kept and used by humans can be derived. These improvements rest in large part on the shoulders of female scientists, working on basic and applied research projects now and in the future for the benefit of the animals.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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ORIGINAL RESEARCH
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Efficacy of Tricaine (MS-222) and Hypothermia as Anesthetic Agents for Blocking Sensorimotor Responses in Larval Zebrafish

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Tricaine, or MS-222, is the most commonly used chemical anesthetic in zebrafish research. It is thought to act via blocking voltage-gated sodium channels, though its mechanism of action, particularly at the neuronal level, is not yet fully understood. Here, we first characterized the effects of tricaine on both body balance and touch responses in freely swimming animals, before determining its effect on the neural activity underlying the optokinetic response at the level of motion perception, sensorimotor signaling and the generation of behavior in immobilized animals. We found that the standard dose for larvae (168 mg/L) induced loss of righting reflex within 30 seconds, which then recovered within 3 minutes. Optokinetic behavior recovered within 15 minutes. Calcium imaging showed that tricaine interferes with optokinetic behavior by interruption of the signals between the pretectum and hindbrain. The motion sensitivity indices of identified sensory neurons were unchanged in larvae exposed to tricaine, though fewer such neurons were detected, leaving a small population of active sensory neurons. We then compared tricaine with gradual cooling, a potential non-chemical alternative method of anesthesia. While neuronal tuning appeared to be affected in a similar manner during gradual cooling, gradual cooling induced a surge in calcium levels in both the pretectum and hindbrain. This calcium surge, alongside a drop in heartrate, is potentially associated with harmful changes in physiology and suggests that tricaine is a better anesthetic agent than gradual cooling for zebrafish laboratory research.

Keywords: zebrafish, animal welfare, 3R, tricaine, gradual cooling, anesthesia, MS-222, optokinetic response

INTRODUCTION

Zebrafish are one of the most commonly used model organisms in biological research; it was suggested in 2017 that more than 5 million zebrafish were used annually (1), and that number has only continued to grow. Understanding animal welfare is crucial to the ethical foundations of such animal experiments, and much work has been devoted to further improve their planning, conduct, reporting and assessment (2–6). For decades, the 3R Principle of animal research (replace, reduce, refine), first introduced by Russell and Burch (7) has formed the core of such efforts, and to this day informs legal regulations of animal research. While several definitions of refinement exist (8, 9), it generally refers to reducing the harmfulness of procedures thus minimizing the suffering of individual animals (4). This includes, but is not limited to, pain (8). While pain and methods to avoid or relieve it are well-understood in some species, they are not in others, including aquatic species (10, 11). Nonetheless, precautions have long been taken to putatively reduce suffering, and one of the most frequent treatments of larval zebrafish is the application of anesthetic agents in preparation for invasive procedures or as a method of euthanasia. Currently, tricaine is the most commonly used laboratory anesthetic, used by 80% of research labs responding to a survey carried out by Lidster et al. (1); despite this fact there has been little investigation into how tricaine acts at the neuronal level in zebrafish. It has been assumed that tricaine preferentially blocks neural signaling in the brain (12), as has been shown in *Xenopus laevis* (13), however, there is a dearth of evidence confirming this claim in zebrafish.

Additionally, multiple studies have found tricaine to be aversive in adult zebrafish, with zebrafish tending to avoid areas where tricaine is present (14, 15). In one of these studies, zebrafish changed their preference in a light/dark box paradigm, from the preferred light side to the non-preferred dark side when tricaine was added (15), showing that this aversion is strong enough to override innate behaviors. It has been noted however, that aversion does not always equate with nociception (16), and the level of animal suffering associated with tricaine administration is thus still unclear.

Any agent that produces a complete or partial loss of feeling can be considered to be an anesthetic, including non-pharmacological agents. Exposure to cold temperatures has recently been observed to have an anesthetic effect in larval zebrafish (17). Gradual cooling has therefore been proposed as an alternative, non-pharmacological anesthetic in zebrafish, but there are comparatively few studies discussing its use, most of which were carried out in adults (18, 19). Similar to the case with tricaine, both of these studies tested the efficacy of gradual cooling at a behavioral level only, finding it to be an effective method of anesthesia. Collymore et al. (18) found that neither tricaine nor gradual cooling led to signs of distress when observing both induction and recovery in adult zebrafish. Behavioral responses during cold treatment are modulated by exposure to analgesics (17), suggesting that nociception could play a role in the overall behavioral reduction observed in cold exposed larvae in the absence of analgesics. Though brain activity in zebrafish is thought to be reduced at low temperatures (20), neural recordings confirming this assumption and characterizing brain responses during anesthetic treatment are missing.

Here, we characterized behavioral, physiological, and neural responses during tricaine treatment and gradual cooling in order to evaluate the possible use of gradual cooling as a method of anesthesia. We assessed the righting reflex and touch responses in freely swimming animals, as well as the neural activity underlying the optokinetic response (OKR) and resultant eye movements in immobilized animals. The OKR consists of reflexive eye movements, the generation of which depends on motion-processing neurons in the visual pretectum and oculomotor neurons in the hindbrain. The investigation of pretectal and hindbrain responses allowed for a direct comparison of sensory and premotor responses to identify at what level along the sensorimotor pathway the necessary activity patterns are lost. We found that tricaine exposure led to a loss of the righting reflex as well as a suppression of reflexive eye movements, though it had only a marginal effect on heartrate. At the neuronal level, tricaine exposure reduced the number of stimulus-associated neurons detected in both the pretectum and hindbrain. While the reduced subset of pretectal neurons exhibited the same tuning as found before treatment, oculomotor-related oscillating hindbrain activity was virtually absent. Gradual cooling, in comparison, had profound effects on evoked eye movements and heartrate, and most critically, induced a surge in calcium levels in both the pretectum and hindbrain. Such calcium waves have previously been associated with apoptosis in larval zebrafish (21). Thus, these results suggest that while gradual cooling may induce a comparable level of anesthesia to tricaine, it has the potential to be more detrimental to the overall health of the animal and the recovery period may, therefore, be inherently more stressful.

MATERIALS AND METHODS

Animals

Animal experiments were performed in accordance with licenses granted by local government authorities (Regierungspräsidium Tübingen) in accordance with German federal law and Baden-Württemberg state law. Approval of this license followed consultation of both in-house animal welfare officers and an external ethics board appointed by the local government. 5–7 days post fertilization (dpf) heterozygous Tg (*elavl3:nls-GCaMP6s)mpn400* zebrafish larvae were used (22). All zebrafish used were also homozygous for the *mitfa* mutation (23). Zebrafish were reared at 29°C on a 14/10 light/dark cycle. Larvae were raised in standard E3 medium containing methylene blue (10^{-5} % v/v) until 3 dpf, when they were sorted for transgene expression and transferred to E3 medium devoid of methylene blue.

Anesthetics

In order to carry out experiments using tricaine, veterinary-grade tricaine was purchased from PharmaQ (Tricaine Pharmaq 1,000 mg/g). Tricaine was prepared at a concentration of 4 g/L in E3 medium (which did not contain methylene blue) and buffered using 1M Tris (pH 9; 4% v/v) to pH7.

During setup development we found that freezing of the E3 media in the petri-dish may be a problem due to the small volumes of liquid used. To prevent freezing, 1% v/v 1,2-propanediol was added to the E3 medium used in these experiments. Though this only led to minimal reductions in the freezing point (<1°C), this (together with setup improvements) was sufficient and no freezing was seen in any experiments. Control experiments were also carried out at this concentration (referred to as vehicle control throughout). This level has been shown not to be toxic to larval zebrafish (24, 25).

Exposure to either tricaine or gradual cooling had a duration of 7 min, and larvae were exposed only once to a single anesthetic

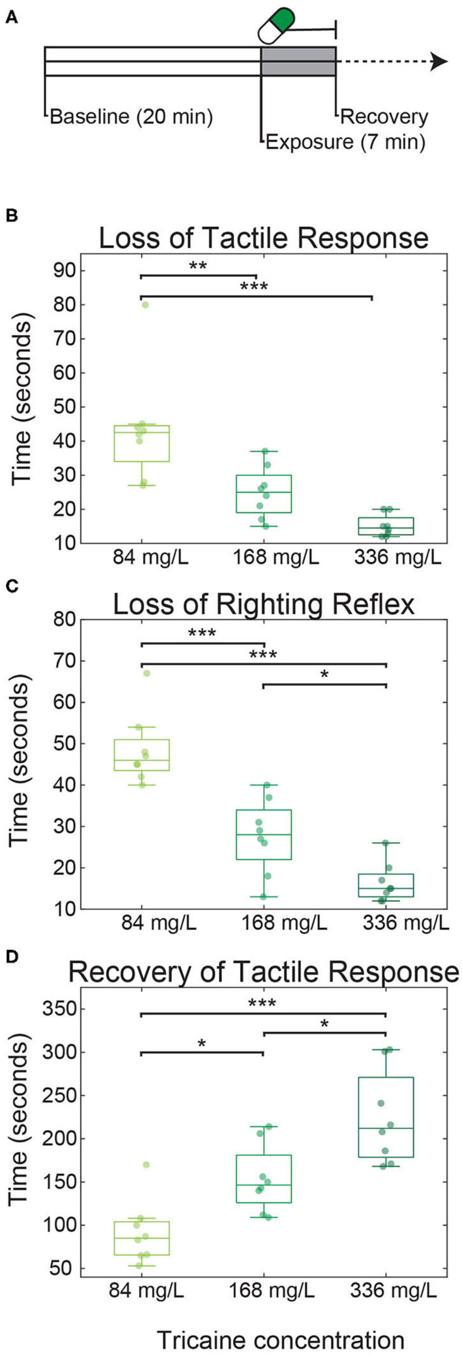

FIGURE 1 | Tricaine onset and recovery in non-restrained zebrafish larvae. **(A)** Larvae were placed in dishes for 20 min to acclimatize (referred to as baseline), followed by 7 min of tricaine exposure. After these 7 min, larvae were transferred to a dish containing tricaine-free E3 and recovery time was assessed. Time to loss of tactile response **(B)**, loss of righting reflex **(C)**, and recovery **(D)** of tactile response were measured in larvae exposed to three concentrations of tricaine: the standard dose (168 mg/L), half this concentration, and double this concentration. Tricaine onset and recovery was dose-dependent, with lower concentrations having the slower onset and faster recovery times. Box plots show quartiles; whiskers extend to the most extreme data points not considered outliers (within 2.7 standard deviations). Results were analyzed via one-way ANOVA with Tukey's HSD test. $N = 8$ for all three conditions, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

agent. All experiments involving embedded fish included a 6 min baseline period immediately before application of anesthesia.

Tactile Tests

Thirty five millimeter petri dishes were filled with 4 mL of E3, then either 168 µL (for control, 0.5X or 1X tricaine conditions) or 336 µL (for 2X tricaine) was removed from the dish, depending on the condition being tested. Individual larvae were placed in dishes and allowed to habituate for 20 min (see **Figure 1A**). After this habituation period, tricaine was added to the dish (bringing the volume back to 4 mL), and the time required for larvae to lose their righting reflex and cease responding to a tactile stimulus was recorded. The tactile stimulus used was a tap to the tail using a mounting needle. After seven min of tricaine exposure, the fish was transferred to a fresh petri dish containing 4 mL E3, as a first wash step, and then to a second dish, again containing 4 mL E3. The time taken for tactile response to occur was recorded. Data was analyzed via one-way ANOVA with Tukey's honest significant difference (HSD) test, $n = 8$ for all conditions.

Behavioral OKR Testing

A modified stage was used in order to deliver anesthetic to the larvae (**Figure 2A**, **Supplementary Figure 1**). The stage was made from aluminum, with two rails (M-SP-3, Newport, Irvine, CA, USA) onto which custom-made aluminum blocks were loaded, which functioned as heat-sinks. A circular Peltier element (TEC-15,2-6,0-51,0-71-51/9-RCH, Minkin Arctic TEC Technologies, Dortmund, Germany), with a hole in the middle was placed on top of the set-up. This hole allowed the eye movements and heartbeat frequency to be recorded from below. A modified glass-bottomed petri dish was fastened above the Peltier element (GW5040B-01, Plano, Wetzler, Germany). Hot glue was used to modify Petri dishes such that E3 medium could flow through only a small channel across the dish. Two tube connectors were glued to each side of the petri dish, and E3 medium was constantly added on one side of the dish and removed on the other side of the dish (P-801, Techlab, Braunschweig, Germany). The connectors removing E3 medium were placed at a shallower angle than those which added E3 to ensure a column of E3 medium remained at all times (~15° vs. ~30°). The E3 medium was provided by a peristaltic pump (ISM4408, Reglo Ismatic Digital, Cole-Palmer, Wertheim, Germany) with a flow rate of 15 ml/min. An overview of anesthesia application is shown in the schematic in **Figure 2A**. For experiments where tricaine was used, one tube supplied E3, another supplied E3 mixed with a known concentration of tricaine, and the other two were used for removal of E3 from the dish. As previously stated, for all cooling experiments the E3 used contained 1% v/v 1,2-propanediol (141545.1211, AppliChem, Darmstadt, Germany) to prevent freezing of the E3 medium at lower temperatures. For these experiments, one tube supplied E3 heated to 60°C, the other supplied cold E3 from a beaker containing ice made from E3 medium, again two tubes were used to remove E3. After passage through the tubes, which resulted in passive warming of the cold E3 and passive cooling of the hot E3, the solutions arrived and mixed in the dish. Temperature was tracked in real time using a Ni-Cr temperature probe and digital

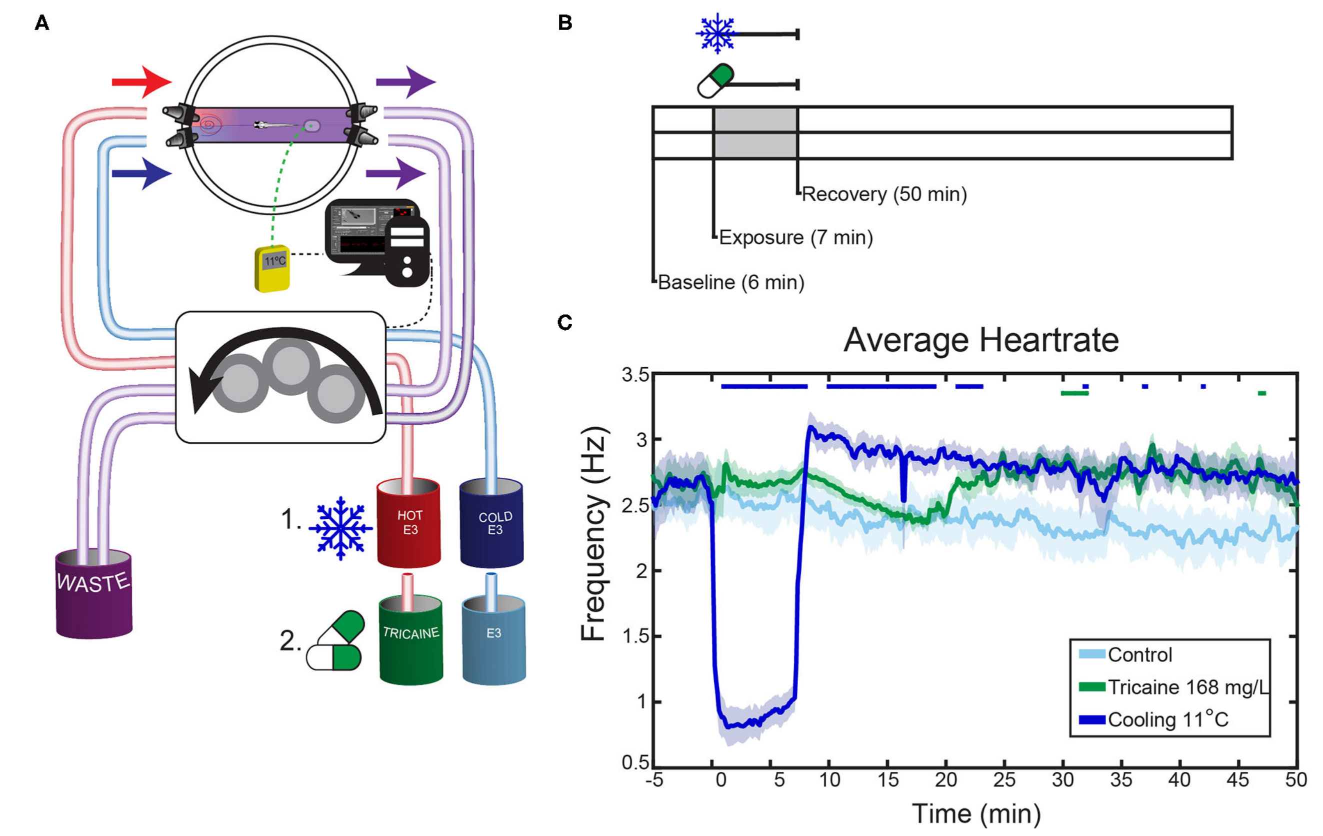

FIGURE 2 | Effects of tricaine and gradual cooling on the heartrate. **(A)** Schematic overview of the experimental setup. Fish were agarose-mounted in glass-bottom petri dishes which were modified to allow liquid to flow through only a narrow channel. The dishes had two connectors on each side in order to apply the liquids, and a sticker to ensure consistent placement of the thermal probe. During gradual cooling experiments, hot and cold E3 were dynamically applied via a peristaltic pump into a dish, where they mixed to achieve the desired temperature under control of a temperature feedback loop (see Methods). In tricaine experiments, the peristaltic pump applied tricaine at the standard concentration (168 mg/L), which was then washed out with drug-free E3. Hot and cold E3 beakers also contained 1% v/v 1,2-propanediol. **(B)** The experimental protocol consisted of 6 min baseline recording, 7 min tricaine or gradual cooling application, and 50 min of recovery. **(C)** The heartrates of larvae exposed to a standard tricaine concentration (168 mg/L), gradual cooling (11°C) and control fish. Average heart rates are shown, the SEM is shown as shaded envelope. Bars above time points indicate significant differences vs. time-matched control larvae (Tukey's HSD test, for clarity exact p-values are not shown, all $p < 0.05$, $n = 7$–8).

thermometer, and sent via a DAQ device in order to dynamically control the temperature (NI USB-6008, National Instruments, Austin, TX, USA).

The Peltier element was used during behavioral OKR and heartrate testing, but not during calcium imaging experiments. The same 5V DAQ device was also connected to three LED drivers (RCD-24-0.35/PL/B, Recom, Gmunden, Austria) which were connected in parallel and powered the analog dimming function and thus altered the current supplied to the Peltier based on the desired temperature. The power supplied to the Peltier was kept constant throughout the 7 min exposure period. The Peltier was only connected to a power supply during the cooling periods.

Removable aluminum blocks were used as heatsinks during the experiment. The blocks were kept in the freezer at −20°C and two blocks were added to the setup immediately before the beginning of the experiment. In the case of behavioral OKR and heartrate experiments, these blocks were exchanged every 20 min. This was not possible during calcium imaging experiments, and instead the blocks were exchanged during the pause between the first and second recording. The addition of the blocks lead to a reduction in temperature of the set-up prior to the start of the experiment and many of the fish were briefly exposed to temperatures of 16–20°C during the alignment of the fish. As this only occurred during the experiments where cooling was carried out (i.e., cooling experimental and cooling control groups), and since we did not observe any significant differences between baseline values for the four conditions (2 controls and 2 treatments), we assume it is unlikely that the early addition of the blocks influenced our findings.

The program ZebEyeTrack was used to control the peristaltic pump while also detecting and tracking the eyes, and displaying visual stimuli using an LED visual stimulus arena (26). The visual stimulus used during behavioral experiments consisted of a moving bar stimulus with a spatial stimulus frequency of 0.033 cycles per degree and a temporal frequency of 18°/s. The stimulus had three phases: 1 min counter-clockwise rotation, 1 min clockwise rotation, and 1 min of 4 s alternations between these two directions (see **Figures 2B**, **3A**). The data was binned in 3 min increments and analyzed based on the number of saccades occurring, and the dynamic range of the eye movements observed. The dynamic range was calculated as the difference between the leftmost and rightmost extreme eye position. Significance was determined via 2-way repeated measures ANOVA with Tukey's honest significant difference (HSD) test, $n = 4$–18.

Heartrate Measurement Alongside OKR Testing

Experiments were carried out as described above, though with a shorter format; recovery was recorded for 50 min only (see **Figure 2B**). During these experiments, the camera below the fish recorded a video of the heartbeat throughout the complete experiment. The frame rate of these recordings was 7–15 frames per second (fps). Videos were rotated in the image processing package Fiji (27), kymographs were generated for both the heart, and another area of the video in order to capture both the heart rate and background noise. The background noise signal was removed from the heartrate trace via an adaptive recursive least square filter. The heartrate signal was then resampled to a constant framerate of 10 Hz and filtered using a high pass filter with a cut-off frequency of 0.5 Hz (visual observation confirmed that there was no cessation of heartrate during any treatment). The Fourier synchro-squeezed transform of the trace was then calculated (Matlab: *fsst*); a simple Fourier transform was not performed due to the irregular sampling rate and the expected variability in heartrate during the recordings. The temporal frequency ridge was then extracted to determine the maximum energy frequency of the recording. Smearing was observed in the temporal frequency ridges immediately after treatments were applied. In order to remove this smearing, an inverse Fourier synchro-squeezed transform (Matlab: *ifsst*) was performed using the highest energy components of the signal (Kaiser window 256, $\beta = 10$), and the power spectrum of the resulting reconstructed signal was calculated (Matlab: *pspectrum*). The temporal frequency ridge of this reconstructed signal was taken as the heartrate (Matlab: *tfridge*). Controls were pooled; 2 unaltered-E3 controls were recorded, 4 controls with E3 containing 1% v/v 1,2-propanediol were recorded. Data was analyzed via 2-way repeated measures ANOVA with Tukey's honest significant difference (HSD) test, $n = 7$–8.

Calcium Imaging Alongside Visual Stimulation

The two-photon imaging path and LED visual stimulation arena have been previously described in Brysch et al. (28). Calcium imaging was performed using a MOM microscope (Sutter Instruments, Novato, CA, USA), Coherent Vision-S Ti-Sa laser and a 20x/1.0 objective (Zeiss W Plan-Apochromat, Jena, Germany). Imaging was carried out at a frequency of 2 Hz, a magnification of 2x in the pretectum, and 1.3–1.5x in the hindbrain, and at a wavelength of 920 nm. Only one plane was imaged per fish per experiment. This was followed by sequential imaging of each brain structure in planes along the optical axis (z-axis); images were taken in intervals of 0.88 μm covering the brain volume 40 μm above and 40 μm below the target image plane (z-stack). $N = 52$ total fish were imaged.

For these experiments the optimal temperature, as determined from behavioral experiments to be 11°C, was tested against the standard tricaine concentration of 168 mg/L. Separate controls were used for comparison; a drug-free control group was tested vs. tricaine, and a vehicle (1% v/v 1,2-propanadiol) control group was compared with the cold treatment. For each experiment, an initial 2 min period of spontaneous activity was recorded, followed by a 6 min period of visual stimulation pre-anesthesia recording, this was then followed by a 7 min period of anesthesia alongside visual stimulus, and another 6 min period during which the anesthetic was removed alongside visual stimulus to show the initial recovery. After a 12 min break a subsequent 6 min recording was made, followed by a 13 min break, and another 6 min recording. A moving-bar visual stimulus was shown during these recordings, but remained stationary during the breaks (see **Figures 4A**, **5A**).

Visual stimulation experiments were carried out alongside calcium imaging, using two distinct stimulus protocol paradigms, one aimed to identify and characterize sensory neurons and the other to do this for motoneurons (see **Figures 5B,C**).

During analysis, we observed that in the neural recordings, where gradual cooling treatment was used, the plane of the recordings underwent a drift in the z-plane, likely owing to the cooling and heating of the aluminum stage. The linear thermal expansion coefficient of aluminum is $\sim 23^{*}10^{-6}$/K, therefore for a stage of this height (15 cm) a 1°C change in temperature would result in a shift of 3.45 μm. Additionally, there was a dramatic alteration in the appearance of the frames, which may have been due to cellular swelling and could alter the pixel identities of neurons in the recording. As these effects were exclusively observed during cooling treatment, this could have caused us to misattribute changes in firing patterns which were due to cells drifting or expanding as being caused by anesthetics. To minimize this possibility, rather than analyze recordings *in toto* we instead broke them up into smaller fragments of 60 or 75 s (for motor hindbrain and sensory pretectum recordings, respectively). Regions of interest (ROIs) were then automatically generated in each fragment in order to identify stimulus encoding neurons in both sensory and motor brain areas. ROIs consisted of 15–25 spatially contiguous pixels whose z-scores were highly correlated with the chosen regressor.

Pretectal Imaging

Imaging was carried out in the pretectum, directly beneath the dorsal boundary to the optic tectum, in order to look for deficits in sensory signals during anesthesia. The visual stimulus consisted of a 7 s exposure to a moving bar stimulus, interspersed with a 12 s pause (see **Figure 5B**; sensory paradigm). After each pause, stimulus direction (clockwise, counter-clockwise) reversed. This stimulus was repeated throughout the recordings, but was not shown between recordings. All video fragments

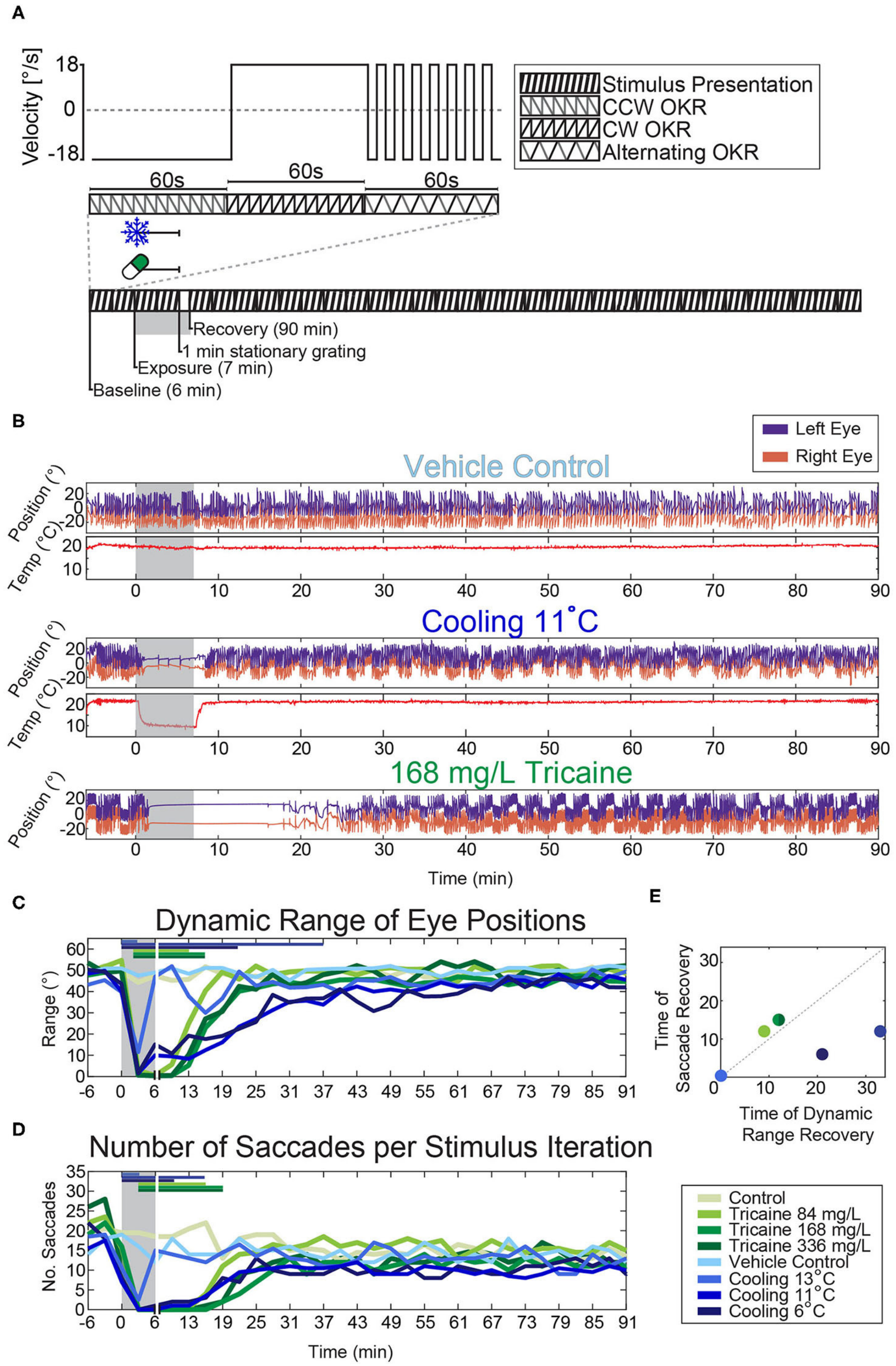

FIGURE 3 | Effects of tricaine and gradual cooling on the optokinetic response. **(A)** Illustration of the visual stimulus (top) and the experimental protocol (bottom). A 3 min looping stimulus was presented to animals using an LED arena (**Supplementary Figure 1**). It consisted of a moving bar rotating at a constant velocity (18°/s) for 60 s counter-clockwise, followed by 60 s clockwise, and finally alternating between counter-clockwise and clockwise in 4 s intervals for a total of 60 s. **(B)** Sample traces of the eye movements evoked by the stimulus protocol during experiments carried out in the presence of the vehicle control (1% v/v 1,2-propanediol), during gradual cooling (11°C) and in the presence of tricaine at a concentration of 168 mg/L. **(C,D)** The dynamic range of the evoked eye movements and the number of saccades occurring during each 3 min stimulus period were analyzed and significance was determined via two-way repeated measures ANOVA with Tukey's HSD test. Horizontal colored bars indicate which time points were significantly different from respective controls, for clarity only one significance level is shown ($p < 0.05$). Vertical white lines indicate the 1 min stationary grating period shown in **(A)**, as no visual stimulus was present during this time. **(E)** Comparison of the recovery timepoints of saccade rate and dynamic range for the different treatments. $N = 4$–18.

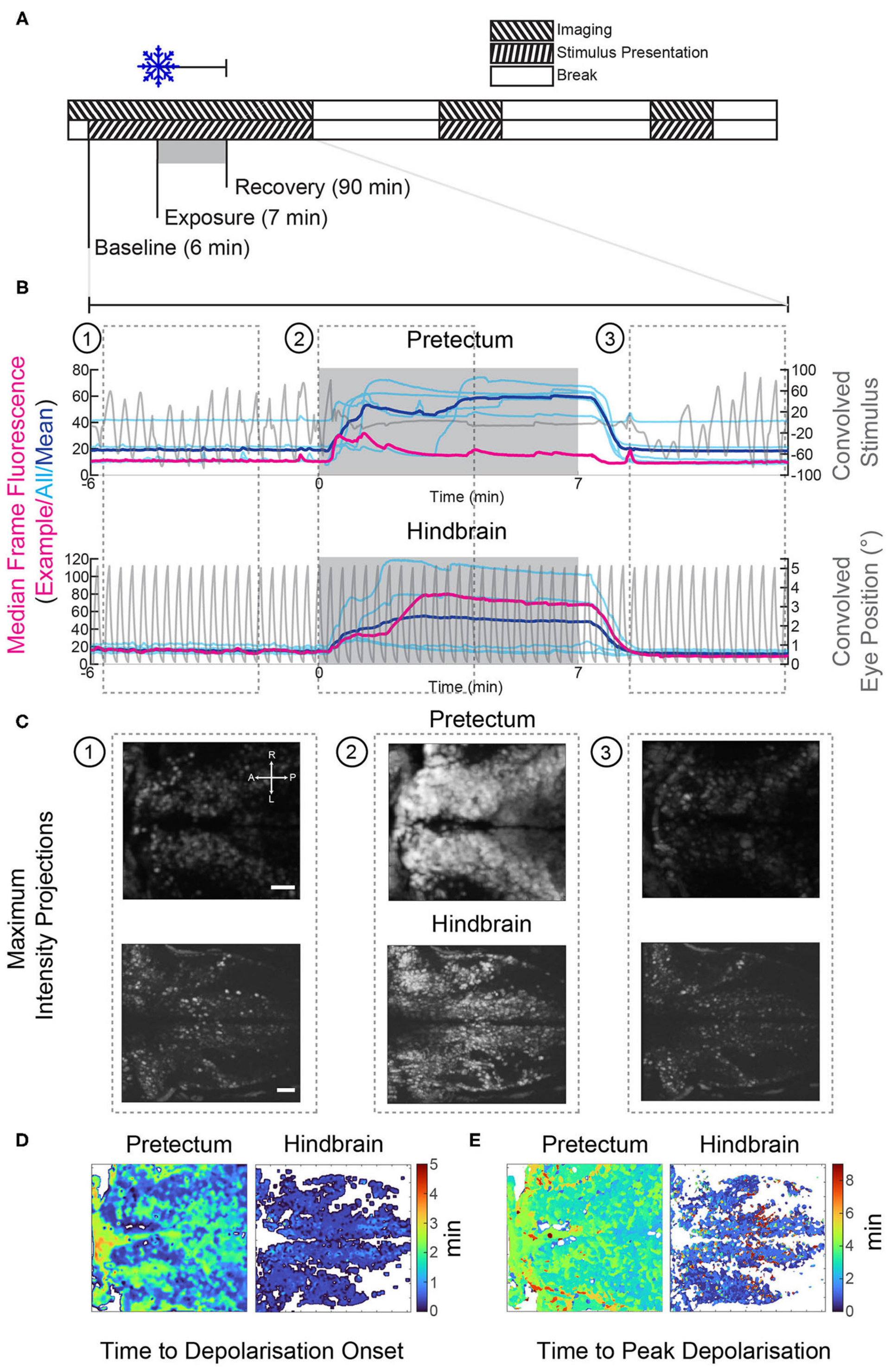

FIGURE 4 | Gradual cooling induced a calcium surge in both the pretectum and hindbrain of exposed larvae. **(A)** Stimulus and imaging paradigm for calcium imaging experiments carried out in both the pretectum and hindbrain. **(B)** Median frame fluorescence for pretectal and hindbrain recordings; traces for individual recordings are shown in cyan and the mean values are shown in blue. The fluorescence trace for a single recording in the pretectum and hindbrain is shown in red, the convolved eye trace and convolved stimulus for the example recordings are also shown in gray. **(C)** Maximum intensity projections at different time points from the example recordings shown in red in **(B)**. 1: before cooling, 2: during cooling to 11°C, 3: recovery 1:30 to 5:40 min after cooling ceased. Scale bars: 30 μm. **(D)** The onset of the calcium surge was calculated in stimulus-correlated (regressor score >0.5) pixels. This onset was defined as the first time point at which the fluorescence was >5 standard deviations above the baseline. **(E)** The time point at which correlated pixels reached their highest pixel intensity. A, anterior; P, posterior; L, left; R, right.

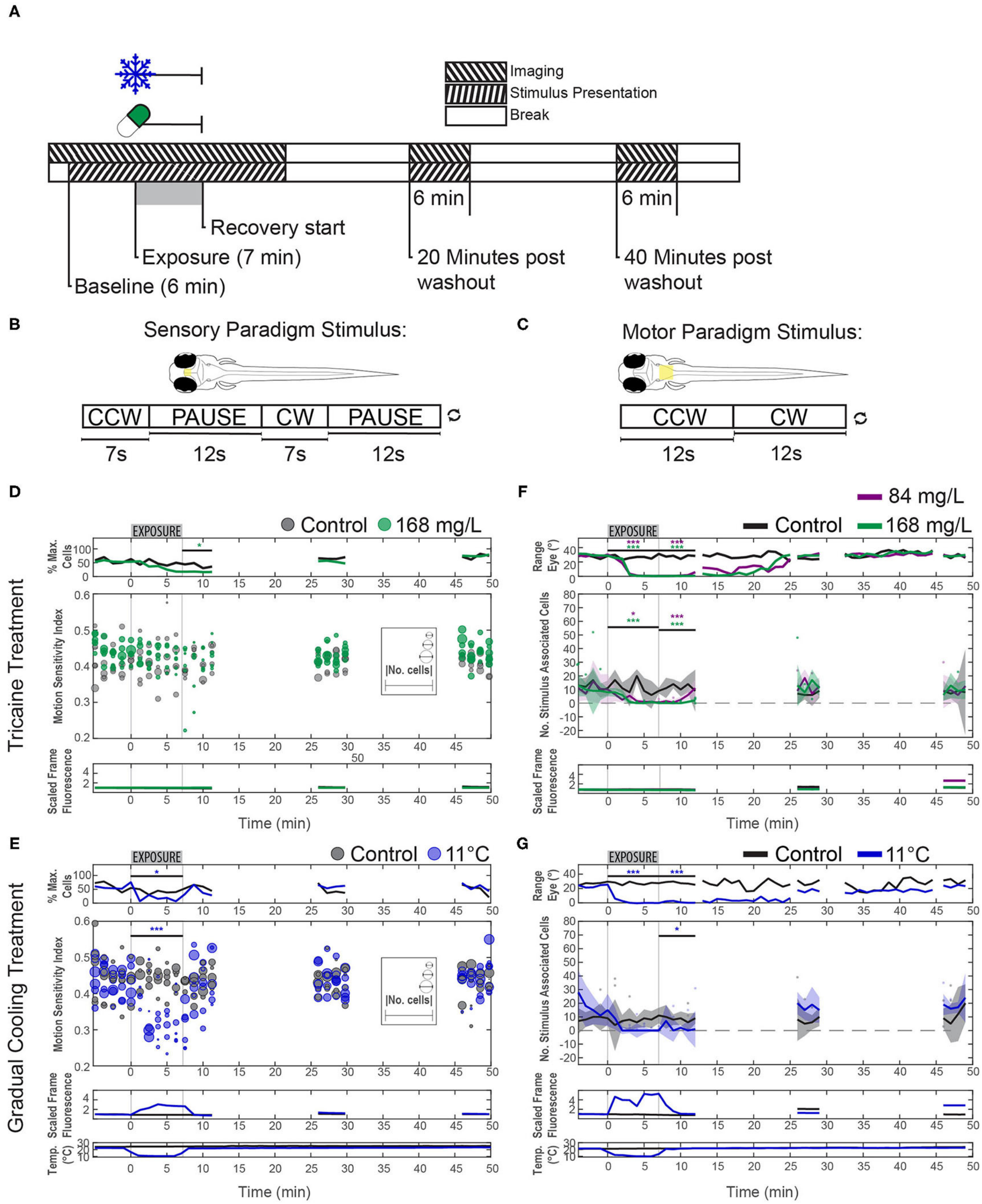

FIGURE 5 | Effects of tricaine and gradual cooling on sensory and motor brain areas. **(A)** The duration of drug exposure was the same as in our previous experiments. Stimuli were presented alongside calcium imaging, and ceased between recordings, as shown in the schematic. Tricaine (168 mg/L and 84 mg/L) and gradual cooling treatments were applied for 7 min (Exposure). **(B)** During imaging of the pretectum, the stimulus consisted of alternating moving (7 s) and stationary (12 s) periods in order to determine the motion sensitivity of identified neurons. **(C)** For experiments in the hindbrain, the stimulus was constantly rotating (18°/s) and alternated between clockwise and counterclockwise motion every 12 s. **(D,E)** Motion-sensitivity of neurons detected in larvae exposed to tricaine and gradual cooling. Note that cooling treatment reduced the number of detected motion-sensitive cells and their motion-sensitivity, while the corresponding effects during tricaine treatment were less pronounced or absent. In the upper row (% Max. Cells), the number of detected neurons is expressed as a percentage of the maximum number of neurons detected via pixel-wise regressor correlation in any minute of the recording. The absolute number of identified cells in individual recordings is shown via the diameter of the data points. **(F,G)** The number of stimulus-associated neurons detected in the hindbrain of larvae exposed to either tricaine or gradual cooling was decreased. The dynamic range of eye positions is shown in the top row. Plots in **(D–G)** differ in style, because in **(D,E)** we assessed two parameters (motion sensitivity and number of cells, each represented by the circles) and only one parameter (number of cells) in **(F,G)**. Results were binned per phase into baseline, treated, recovery, recording 2 and recording 3 and analyzed via two-way repeated measures ANOVA with Tukey's HSD test, $n = 4–7$. $^{*}p < 0.05$, $^{***}p < 0.001$.

underwent analysis with a previously published method in order to detect ROIs based on pixel-wise correlation with a stimulus encoding regressor (28, 29). The motion sensitivity index of all identified ROIs was calculated based on the activity of the neurons during moving vs. stationary stimulus periods for two full stimulus presentations; if the index fell below 0.1 in more than two of the four iterations the ROIs were excluded from further analyses. This step was implemented to control for false positive motion responses caused by a thermally induced calcium surge.

The first four of the final 5 s of the stimulus presentation were used (t_{end-5}: t_{end-1}).

The calcium signal DFF at time point t was calculated as the difference between the calcium indicator fluorescence and the baseline calcium indicator fluorescence (Fb; defined as the mean of the lowest 25 values in the ROI trace) divided by Fb:

$$DFF\,(t) = \frac{F\,(t) - Fb}{Fb} \quad (1)$$

The motion sensitivity index of the j^{th} ROI was calculated as follows:

$$MSI_j = \frac{\sum_t DFF_j on(t) - DFF_j off\,(t)}{\sum_t DFF_j on\,(t) + DFF_j off\,(t)} \quad (2)$$

where DFF^{on} (t) and DFF^{off} (t) correspond to the calcium signal during static and moving stimulus presentation, respectively.

Data was analyzed via two-way ANOVA with Tukey's honest significant difference (HSD) test, $n = 5$–6.

A control experiment was carried out during which larvae were recorded for 6 min prior to the addition of tricaine followed by exposure to 168 mg/L tricaine for 15 min. The visual stimulus was shown throughout. A 1-way ANOVA was carried out on detected ROIs across 75 s time-bins on the resulting motion sensitivity values, the results of which were not significant, $n = 4$. A 1-way ANOVA on the number of ROIs detected across time-bins was highly significant ($p < 0.001$). This control experiment showed that a longer treatment period (15 min) had similar effects as the shorter treatment period used in all other recordings. In both type of experiments, tricaine reduced the number of detected ROIs, but not the motion sensitivity of these remaining ROIs. Therefore, the finding of remaining motion sensitivity does not appear to be related to the shortness of our treatment period.

Hindbrain Imaging

Imaging was carried out in the zebrafish hindbrain in order to detect deficits in motor signals during anesthesia. Recordings were carried out in the plane corresponding to the location of the Mauthner cell somata and extending rostrally to the cerebellum and caudally to the spinal cord, the imaging region extended dorsally for 20 μm. The visual stimulus consisted of a moving bar stimulus, which alternated between clockwise and counterclockwise every 12 s, but was not shown between recordings (see **Figure 5C**). The analysis of motor encoding activity was complicated by the loss of eye movements during anesthesia. In order to determine whether residual motor encoding signals were present during periods where no behavior was seen, our approach focused on neurons whose firing was modulated by the stimulus. Due to the sinusoidal nature of the stimulus, a fast Fourier transformation was carried out on the detrended DFF traces, and the number of ROIs which had a peak at the stimulus frequency was counted. Changes in the number of identified cells were analyzed via two-way ANOVA with Tukey's honest significant difference (HSD) test, $n = 4$–7. Wilcoxon rank-sum tests were used to determine changes in the median dynamic ranges as data were found to be non-parametric.

Cross-Correlation Structures

ROIs detected in each time bin were ordered based on their correlation with the clockwise-stimulus regressor. The resulting matrices were then averaged within each of five treatment stages (pre, during, post and two recovery stages) resulting in five correlation maps per larva. A weighted average was then generated across larvae based on the number of ROIs identified.

Generation of Calcium Surge Heat Maps

Heat maps shown in **Figures 4D,E** were generated for whole recordings of gradual cooling experiments. 8-bit rigid body registered AVI recordings were used. The videos were first filtered using a two-dimensional Gaussian filter ($\sigma = 2$) (Matlab: *imgaussfit*). Individual pixels were then smoothed using a Butterworth filter (1st order, cut-off frequency 0.2 Hz; Matlab: *butter*), as described by Niemeyer et al. (30). Individual pixels were correlated with the median fluorescence trace in order to generate a pixel correlation mask, only pixels with a correlation >0.5 were included in this mask. The onset of the calcium surge was determined as the time point at which the pixel value exceeded five standard deviations above the baseline. A Wilcoxon rank-sum test was carried out in order to determine whether the surge occurred significantly earlier in the hindbrain vs. the pretectum. The onset values for all pixels contained within the masks for all recordings in either the hindbrain or pretectum were collated and the resulting populations were statistically tested against one another. The maximum calcium surge was determined as the time point at which the pixel value was highest. In order to exclude outliers, this value was only included if the threshold was surpassed a second time within 3 s.

RESULTS

Behavioral Effects in Free Swimming Larvae

All larvae exposed to tricaine (**Figure 1A**) rapidly lost their tactile response (**Figure 1B**; median durations of 43 s for a concentration of 84 mg/L, 25 s for 168 mg/L, and 15 s, for 336 mg/L). This was followed quickly by the loss of righting reflex (**Figure 1C**; median durations of 46 s for 84 mg/L, 28 s for 168 mg/L, and 15 s for 336 mg/L) indicating that the fish had reached surgical level anesthesia [stage 3 as defined by Sneddon (31)]. The time to recovery, as assessed by response to tactile stimulation, was 85 s for 84 mg/L, 147 s for 168 mg/L, and 212 s for 336 mg/L. Thus, anesthesia onset was much faster than recovery and both onset and recovery in free swimming larvae were strongly dose dependent as assessed via 1-way ANOVA (tactile response $p < 0.001$; equilibrium $p < 0.05$; recovery $p < 0.001$).

Effects of Anesthetic Agents on the Optokinetic Response

In order to analyze the effects of anesthetics on behavior we assessed the optokinetic response. The optokinetic response is a reflexive behavior and therefore we assume that it is harder to suppress by anesthetic agents than, for example, appetitive behaviors like prey capture responses. Thus, it should provide an accurate readout for the general effects of anesthesia on behavior and brain responsivity. The anesthetic agents were applied via a peristaltic pump. Three tricaine concentrations and three temperatures were tested. In the case of cooling, hot and cold E3 medium, containing 1% v/v 1,2-propanediol, were mixed within the Petri dish to achieve one of the three defined temperatures; a thermometer fixed in the dish allowed for dynamic control of the ratio of hot to cold water in the dish (see **Figure 2A**). In the case of tricaine application, E3 medium containing tricaine at one of the test concentrations was pumped into the Petri dish and later washed out and thereby replaced by medium not containing tricaine (**Figure 2A**).

During each of the experiments the agarose surrounding the eyes was removed and animals were stimulated with a moving grating presented on a surrounding display to elicit an optokinetic response (**Figure 3A**). The effects of each method of anesthesia on the dynamic range of the eye position (**Figure 3C**) and the number of saccades (**Figure 3D**) were assessed. A two-way repeated-measures ANOVA, with Tukey's HSD test was carried out on the resulting data. Both the effects on the dynamic range and saccade numbers were significantly time-dependent ($p < 0.001$ for both tests), and treatment-dependent ($p < 0.001$ for both tests). Larvae exposed to tricaine recovered their dynamic range of eye movements before saccades returned, the opposite was true for gradually cooled larvae (**Figure 3E**).

Tukey's HSD tests found that the dynamic range of eye movements was significantly decreased from about 30° to <10° after 3 min of exposure to either anesthetic (**Figure 3C**, **Supplementary Figure 2**). The onset was faster for the cooling treatments. Considering our earlier results that tricaine is effective within 1 min in free swimming larvae (cf. **Figure 1B**), this difference in effect onset timing is likely due to the time taken for the tricaine to reach an effective concentration in the agarose-embedded larvae. The full recovery of dynamic range occurred faster in tricaine-treated animals (ca. 10 min) than in animals cooled to 11 or 6°C (ca. 15 to 30 min), while larvae cooled to 13°C recovered almost instantly.

In cooled larvae, the saccade rate was reduced to almost zero saccades per minute after 3 min, and appeared to recover within 5 min. The loss of saccades was longer-lasting in larvae treated with the standard tricaine concentration (168 mg/L) than in cooled larvae and remained significant for up to 14 min following removal of tricaine. In cold-exposed larvae, the recovery success was more variable across larvae, whereas tricaine-treated larvae showed less inter-individual variability (data not shown).

Zebrafish Heartrate

The effects of anesthetics on the heart rate were analyzed by applying the two anesthetic agents via a peristaltic pump (**Figures 2A,B**). We chose a temperature of 11°C because it was shown to be effective in the optokinetic response experiments just described. The standard concentration (168 mg/L) of tricaine was used.

These experiments showed that cold and tricaine treatments have temporally distinct effects on zebrafish heart rate (see **Figure 2C**, **Supplementary Figure 3**). Both experimental protocol time and treatment type had significant effects on heartrate as assessed via a repeated-measures ANOVA ($p < 0.001$ for both conditions). Tricaine did not appear to alter the heartrate in the treatment period, which agrees with previous reports (32). However, a heartrate increase was seen during recovery commencing at the time point corresponding to the time at which eye movements (see below) had fully recovered to control levels (∼22 min, see **Figure 2C**), though this was not significant throughout most of the recovery period. Gradual cooling by contrast had profound effects on heartrate, causing a 60 % drop in heartrate during treatment, followed by a strong and sustained increase (∼15%) during recovery.

Neuronal Signaling in Sensory and Motor Brain Areas

We measured neuronal activity via calcium imaging and used a similar experimental protocol as before, but with a modified visual stimulus protocol (**Figures 5B,C**) to best capture alterations in the responses of neurons in the pretectum and hindbrain.

In all cooling experiments, regardless of brain area, the calcium level of the neurons started to increase drastically within ca. 30 s of cold treatment initiation and then remained elevated (**Figures 4B–E**). Calcium levels dropped once the temperature returned to baseline levels (**Figure 4B**), i.e., within 2 min after the end of the cold treatment period. Fluorescence changes were directly linked to our recorded temperature changes, but slightly delayed (**Figures 5E,G**; bottom). The timing of calcium surge onset was somewhat variable across neurons (**Figures 4B,D,E**), and occurred significantly earlier in hindbrain (mean onset 32 s, standard deviation 28 s) than in the pretectum (mean onset 43 s, standard deviation 35 s) (Wilcoxon rank-sum test, $p < 0.001$). Despite these drastic fluorescence changes, we were able to detect and quantify stimulus-associated neural activity which rode on top of the calcium surge (**Supplementary Figure 4**, also discussed below).

Calcium signals were analyzed in fragments of 60 s each (see Methods) to circumvent artifacts resulting from image drift. The image drift was likely caused by a combination of temperature-dependent changes of the aluminum stage (linear thermal expansion coefficient: 0.002 % per Kelvin) and also morphological changes of the larva induced by the cooling. Tricaine and gradual cooling differed in their effects on sensory tuning (see **Figures 5D,F**, Methods), which we quantified using a motion-sensitivity index and the number of remaining motion-sensitive neurons (see Methods). Next to detailed plots showing results for all individual recordings (**Figures 5D,F**), the main findings are illustrated in the summary plots in **Figures 6A,B**. Treatment had a significant effect on the motion sensitivity index over time (2-way repeated measures ANOVA, $p < 0.05$) and time alone ($p < 0.001$). In gradual cooling experiments, the number of detected neurons with stimulus-associated activity dropped significantly during treatment (Tukey's HSD test, $p < 0.05$), but recovered within 5 min after return to baseline temperature (Tukey's HSD test, $p = 0.6$). During tricaine experiments, the number of detected stimulus-associated ROIs was not significantly altered during treatment (Tukey's HSD test, $p = 0.09$), but became reduced during the first 5 min of recovery (Tukey's HSD test, $p < 0.05$). The number of detected cells was significantly correlated with treatment ($p < 0.001$) and time ($p < 0.001$) in 2-way ANOVA.

In contrast to the change in the number of detectable stimulus-associated neurons, detected neurons still showed normal levels of motion sensitivity during tricaine treatment (no statistical difference observed, $p = 0.78$, **Figure 5D**), and direction sensitivity was also unaltered (data not shown). For the cooling treatment, a reduction in measured motion sensitivity was observed (Tukey's HSD test, $p < 0.001$). This apparent loss of motion sensitivity in the cooling condition was likely—at least in part—caused by the effects of the calcium surge in our analysis. An independent analysis, which relied on image analysis to detect ROIs (33), was performed to exclude potential analysis bias resulting from the calcium surge, also detected stimulus-associated pretectal calcium responses, which appeared to be lost during cooling but to recover afterwards (**Supplementary Figure 5**).

To determine whether the only moderate effects of tricaine treatment on sensory brain activity were due to the short exposure period, four larvae were exposed to 168 mg/L tricaine for 15 min and a 1-way ANOVA was carried out to determine whether the stimulus-associated activity was altered. This test did not find an according significant effect. While the number of detected neurons also decreased over time in these extended recordings, stimulus-associated neurons were found in all but the final minute of one of these four recordings. Thus, tricaine-anesthetized zebrafish show residual sensory brain activity with relatively normal tuning although optokinetic behavioral responses have ceased.

Next, we characterized premotor and motor hindbrain responses in the caudal hindbrain. This brain region contains neuronal populations which drive the lateral and medial rectus extraocular eye muscles, including abducens motoneurons, abducens internuclear neurons and further premotor neurons. We refer to these neurons as premotor from hereon, although motoneurons were likely included in our recordings. Both tricaine and gradual cooling had a pronounced effect on the number of stimulus-modulated neurons detected in the hindbrain (see **Figures 5F,G**, **6C,D**); a 2-way ANOVA identified a significant influence of both time ($p < 0.001$) and treatment condition ($p < 0.001$). This decrease in the number of stimulus-modulated neurons was significant for both concentrations of tricaine during the treatment phase (168 mg/L $p < 0.001$; 84 mg/L $p < 0.05$) but not significant during cooling treatment (11°C). In the first minutes of recovery the number of stimulus-modulated neurons was decreased both for tricaine groups (168 mg/L: $p < 0.001$; 84 mg/L: $p < 0.001$) and for the gradual cooling group (11°C: $p < 0.05$).

There was also a significant effect of treatment on the dynamic range of eye positions (see **Figure 6D**); once again, a 2-way ANOVA identified a significant influence of both time ($p < 0.001$) and treatment condition ($p < 0.001$). This was significant for both tricaine (168 mg/L and 84 mg/L) and cooling treatment (11°C) during ($p < 0.001$) and in the recovery immediately after ($p < 0.001$). These results are in line with the previous behavioral findings (see **Figure 3C**).

Loss of Coordinated Oculomotor Hindbrain Activity in Treated Larvae

In untreated larvae, the hindbrain generated alternating leftward and rightward oculomotor activity patterns associated with optokinetic responses to our alternating directions of stimulus motion. To characterize this premotor activity in treated animals (who had lost their ability to move the eyes), we quantified the pairwise cross-correlation between all neuronal ROIs in the hindbrain. Cross-correlations during each of the treatments differed from those immediately after treatment. **Figure 6E** shows cross-correlation matrices where ROIs were ordered based on their individual correlation to a clockwise-stimulus regressor. During both the spontaneous activity prior to the baseline period (not shown) and the baseline period before anesthesia application, this cross-correlation resulted in a structure with two observable anti-correlated populations (**Figures 6E,F**; red and blue). These anti-correlated populations mainly correspond to eye position drive in the left and right hemisphere of the hindbrain (especially the nucleus abducens), which code for leftward and rightward eye positions, respectively (28). During anesthesia, the neural activity lost its anti-correlational components. In the case of tricaine, there was first an increase in the number of pairs with positive correlations during treatment but immediately after treatment, excluding the diagonal of auto-correlation scores of 1, the positive cross-correlation matrix values were reduced. In comparison, in the case of cooling, negative activity correlations were lost as well for both treatment and early recovery, and most neuron pairs showed weak positive correlations during both treatment and early recovery. The deficit in anti-correlated activity, combined with the near-complete loss of stimulus-associated neurons (**Figures 5F,G**), suggests that during each treatment, the ability of the hindbrain premotor structures to encode bidirectional eye movements is strongly impaired, as the network activity becomes decorrelated.

DISCUSSION

In this study we characterized the effects of two anesthetic agents, tricaine and gradual cooling, on swimming behavior, heartrate, eye movements, and sensorimotor circuits underlying the optokinetic response. We find that while tricaine has a slower onset, both tricaine and cooling are effective methods of immobilization and both suppress sensory and

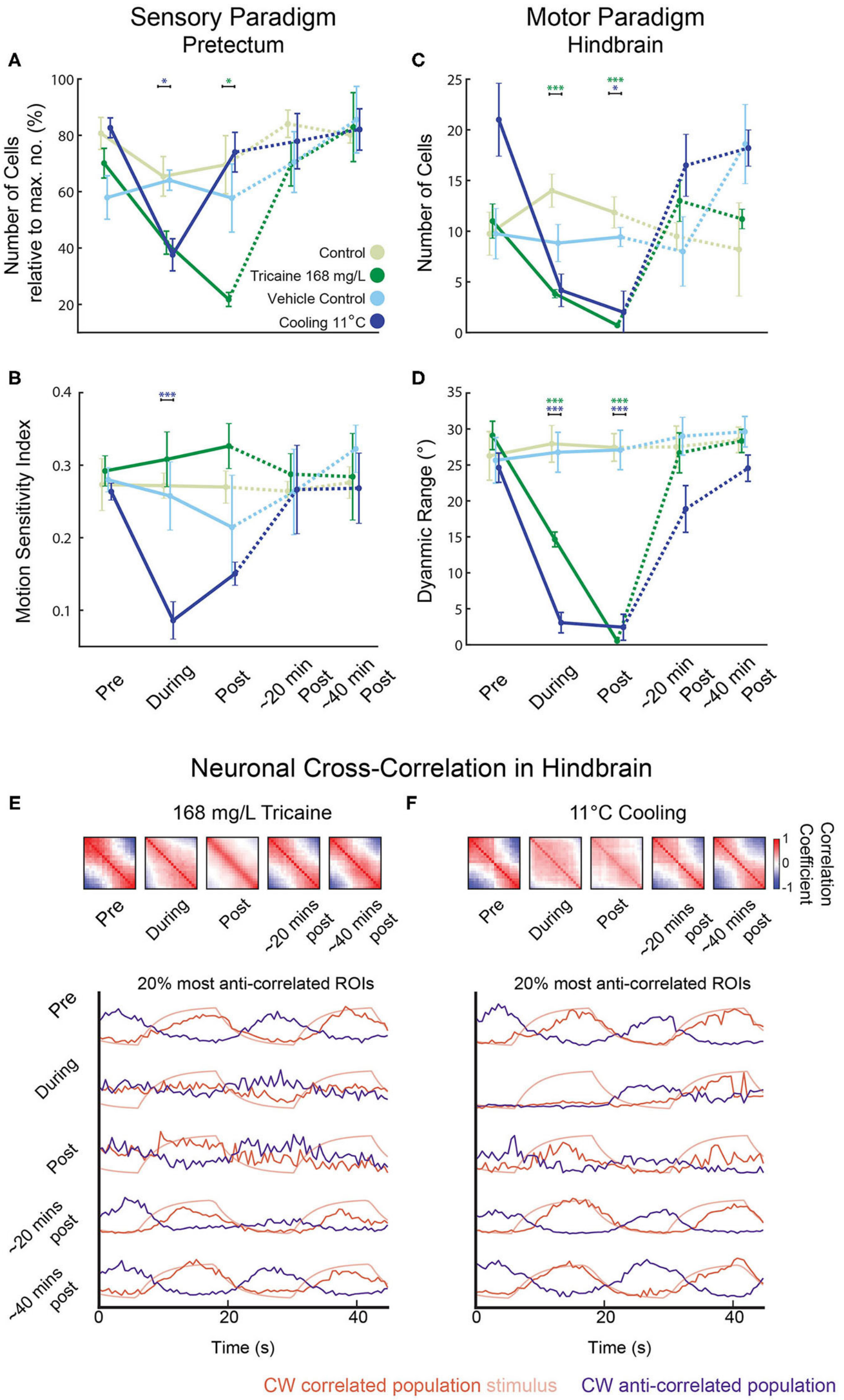

FIGURE 6 | Reduced sensorimotor activity in pretectum and hindbrain after application of tricaine and gradual cooling anesthesia. **(A–D)** Summary of the results from **Figure 5** highlighting the main treatment effects on the number of identified cells in the pretectum **(A)**, the motion-sensitivity index of remaining pretectal neurons **(B)**, the number of identified cells in the hindbrain **(C)**, and the dynamic range of eye movements observed **(D)**. In **(A)**, the number of remaining motion-sensitive neurons relative to the maximal number of motion-sensitive neurons detected in the recording is quantified. Results were binned per phase into baseline, treated, recovery, recording 2 and recording 3 and analyzed via two-way repeated measures ANOVA with Tukey's HSD test, $n = 4$–7. $^{*}p < 0.05$, $^{***}p < 0.001$. **(E,F)** Top: Pair-wise neuronal cross-correlation matrices for the hindbrain of larvae treated with 168 mg/L tricaine or cooled to 11°C. y and x axes of each matrix correspond to the neuronal ROIs sorted according to their individual correlation to the clockwise stimulus. Cross-correlation matrices were calculated for the five different time periods shown and in each displayed matrix, the matrices of all individual recordings and larvae were averaged (a weighted average that took into account the number of ROIs per recording). Bottom: The 20% most correlated (positively or negatively) ROIs are shown. Stimulus traces show expected activity of a CW stimulus-correlated ROI (i.e., the kinetics-adjusted stimulus regressor see Methods).

motor neural activity. But they differ in effects relevant to animal well-being (**Figure 7**). In contrast to tricaine, gradual cooling strongly reduces the heartrate, induces a calcium surge in the brain, permits residual eye movements, and has a comparatively longer recovery period. These results together suggest that tricaine should be considered the preferred anesthetic agent out of these two methods for zebrafish larvae.

Tricaine and Gradual Cooling Have Comparable Effect Onset Times

In freely swimming larvae, a standard tricaine concentration induced loss of tactile response within ca. 40 s (**Figures 1B–D**). For agarose-embedded larvae, eye movements were lost within ca. 1 min in cooled larvae (**Figure 5G**) and ca. 3 min in tricaine-treated larvae. These results together suggest that in freely swimming larvae, the anesthetic effects of both tricaine and cooling manifest within tens of seconds and that the slower onset time for our tricaine in embedded animals was due to the (relatively) slow diffusion of tricaine through the agarose. Previous studies in adult fish have shown the onset of tricaine to be faster than that of gradual cooling (18).

Freely swimming fish regained their ability to respond to a startle-inducing stimulus within 147 s after tricaine (168 mg/L tricaine) wash-out. This recovery was much faster than the ∼11–14 min' recovery seen for optokinetic responses in embedded fish treated with tricaine. The large difference in recovery times suggests that the optokinetic response is more vulnerable to anesthetic agents than the startle response, since the time difference cannot be explained by slower diffusion or removal of tricaine in the embedded preparation.

Tricaine Suppresses the Optokinetic Response More Reliably Than Gradual Cooling

During behavioral experiments, we found that both tricaine and gradual cooling effectively suppressed the optokinetic response. However, while both treatments appeared to have a smooth and rapid onset, the anesthesia in the cooled larvae was much more variable as evidenced by the number of saccades occurring in the second half of the cold exposure. When observing the eye traces of the cooled larvae, approximately a third show convergent and divergent slow velocity eye movements and saccades during the treatment (see **Figure 3B**), which cannot be explained by the stimulus. While these saccades may be true spontaneous saccades, such a preponderance of spontaneous vergence activity is atypical. It is also possible that they were due to surges in neural activity, and are better described as seizure-type behavior. The presence of these spontaneous movements during anesthesia is problematic as they could occur during surgeries or other procedures, and potentially lead to injury.

Cooling to 11°C Is Sufficient to Suppress Reflexive Behavior

The optokinetic response recovered most rapidly from exposure to 13°C, however, incidence of saccades and dynamic range of eye movements were not completely suppressed when compared to treatment with 11 and 6°C temperature (see **Figure 3C**). As we found that 11°C was already effective in suppressing both parameters of the optokinetic response to a similar degree to that of tricaine, we chose to use this temperature in both the heartrate and calcium imaging experiments which followed. Additionally, Collymore et al. (18) observed that an adult fish cooled to 8°C died, while 10°C was an effective temperature. Thus, a temperature of 10–11°C is likely safer for the larvae (though no larvae died at either temperature). While we found that fish exposed to 6°C apparently recovered faster than those exposed to 11°C, a larger sample size for the group tested at 6°C would be needed to confirm this trend.

Gradual Cooling, but Not Tricaine, Decreases Heartrate

The effects of tricaine and cooling on heartrate differed during treatment. Only cooling resulted in a decrease, but both treatments resulted in a post-treatment increase in heartrate during recovery. Contrary to our results, other authors have reported heartrate decrease due to tricaine, which can potentially be explained by differences in study design. Tricaine exposure had previously been shown to maximally decrease heartrate after 10 min (34), which is longer than our total exposure time. Craig et al. (32) only saw significant effects at a concentration of 250 mg/L, much higher than what was tested here (168 mg/L). The decrease in heartrate observed for cooling is likely directly caused by a change in membrane permeability and conduction of the pacemaker cells (35). In line with this, Gierten et al. (36) have shown that temperature is positively correlated with heartrate in zebrafish, although they did not test at low temperatures. Though the sympatho-vagal balance of larval zebrafish has not yet reached its adult state (37), decreases in heartrate have been shown to be sensitive to muscarinic antagonists, consistent with the hypothesis that they are mediated by the parasympathetic nervous system, while increases can be blocked via beta-adrenergic antagonists, consistent with a potential sympathetic drive (38). Therefore, the fact that both treatments induced a tachycardic state following anesthetic removal and corresponding to the time point of recovery of eye movements may suggest that treatments were aversive or stressful for larvae.

Tricaine Treatment Decreases the Number of Stimulus-Associated Neurons in the Pretectum, but Does Not Alter the Tuning of Those Detected

We found that tricaine significantly reduced but did not fully abolish the number of motion-sensitive cells detected in the pretectum, while the remaining tuned cells showed a similar level of motion tuning as cells detected in the baseline period (**Figures 5D**, **6B**). These neurons were detected despite the

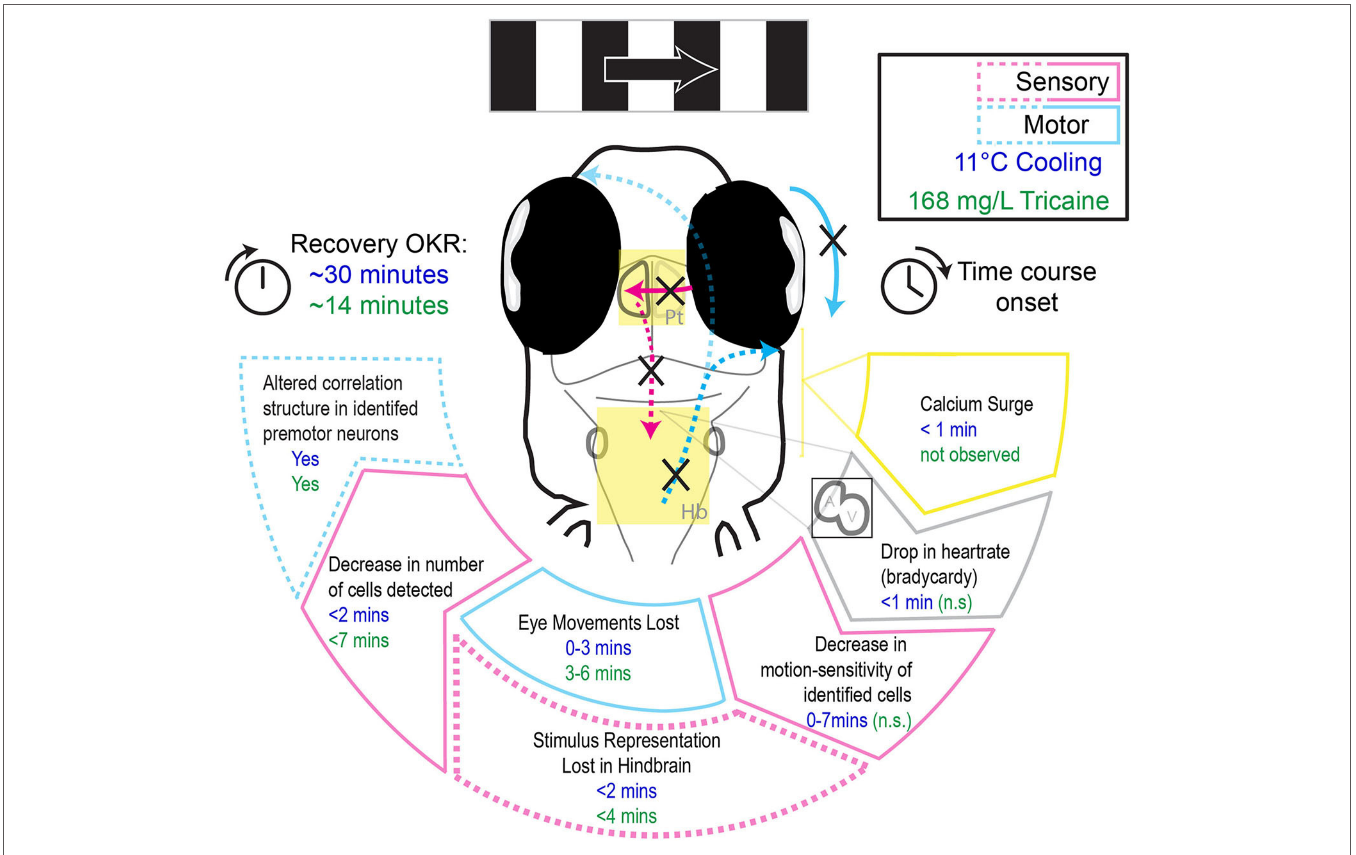

FIGURE 7 | Schematic illustrating the differential effects of 168 mg/L tricaine and 11°C cooling on physiological parameters and the optokinetic response, as well as their respective time courses.

lack of detectable eye movements seen in previous recordings (**Figure 3B**). Thus, during tricaine anesthesia, behavioral responses cease even though visual stimuli are still processed in the pretectum. This result is similar to previous findings in *Xenopus* (13) as well as those of Machnik et al. (39), who found that visual responses were decreased, though not entirely absent in Mauthner neurons in adult goldfish exposed to 100 mg/L tricaine.

Gradual Cooling Induces a Wide-Spread Calcium Wave in Both the Pretectum and Hindbrain

In the case of gradual cooling, the number of identified stimulus-associated neurons was decreased in the pretectum, and in contrast to tricaine, an apparent decrease in the fidelity of remaining encoding pretectal neurons was seen (see **Figures 5E, 6B**). The interpretation of our fluorescence measurements during cooling is potentially strongly affected by the observed calcium surge, which might mask remaining motion sensitivity, or falsely imply motion sensitivity, due to the precise time point of the calcium surge relative to the motion stimulus. We attempted to control for this by requiring all motion-selective neurons to exhibit motion selectivity indices above a threshold of 0.1 in at least two out of four stimulus iterations per time-bin, thus removing neurons which may have had a calcium surge coinciding with motion during just one iteration.

Though the effects of cold on neuronal signaling have been described in other animals (40), to the best of our knowledge, this calcium surge has not been reported for zebrafish elsewhere. However, a spreading depolarization has recently been shown to occur in larval zebrafish exposed to noxious heat (41), and also following extended (>25 min) mechanical suppression of heartbeat (21). Spreading depolarization refers to a slow spreading wave of depolarization which travels through the brain, is similar to a seizure, but occurs at a much slower timescale (2–6 mm/min). This phenomenon is thought to underlie migraines and traumatic brain injuries (42), and may correspond to what we are observing here.

Calcium waves in the hindbrain appeared to both occur earlier and reach their peak levels earlier (**Figures 4B–E**) than calcium waves in the pretectum. However, due to pixel saturation in some recordings, we cannot unequivocally confirm this. While it is possible that the timing difference represents physiological differences between hindbrain and pretectum, it could also be a consequence of the embedding procedure: during hindbrain recordings agarose was removed from around the eyes, thus exposing the brain directly to the cooled medium.

The calcium wave may be caused by energy insufficiency (42, 43). Metabolic processes are known to slow down with decreased temperature, referred to as universal temperature dependence (UTD) (44). Cellular ionic homeostasis relies on a balance between passive and active fluxes. In fish, the temperature coefficient (Q_{10}) of these processes are known to differ, meaning that at colder temperatures either passive flux must be downregulated, or active flux must be rapidly upregulated in order to maintain homeostasis (45). If the rate of active, ATP-dependent, processes falls below the rate necessary to maintain homeostasis, the balance shifts and, without a corresponding alteration in passive flux, this can lead to a depolarizing shift in resting membrane potential intracellular sodium accumulation. The Na^+/K^+-ATPase, which is responsible for sodium extrusion, is the most energy demanding neural process (46), and if neurons do not have sufficient energy, they can no longer maintain osmotic balance. This increased intracellular sodium concentration will then result in cell swelling as water enters the cell via the osmolality gradient (47, 48). It also reduces the membrane potential, and causes the reversal of the Na^+/Ca^{2+} exchanger resulting in Ca^{2+} entry to the cell and eventual depolarization (49, 50). The calcium ions continue to accumulate as the activity of the Ca^{2+} ATPase pump is also decreased, as is ATP-dependent reuptake by the endoplasmic reticulum. Failure of the Na^+/K^+-ATPase pump is thought to be the mechanism behind spreading depolarization, a phenomenon reported to occur during noxious heating of larval zebrafish (41). Similarly, it appears likely that the observed calcium wave in our experiments was caused by insufficient active efflux from neurons during cooling (50). Both decreases in passive flux and increases in active flux have been independently reported in long-term cold adapted fish (45) which may explain why zebrafish can inhabit colder water bodies [down to 6°C according to (51)], however, such adaptations likely occur over much longer time courses than those induced in this study.

ATP depletion has been widely considered to underlie the damaging effects of hypothermia, and the possibility that this is what occurs during cooling in our experiments is supported by three observations. First, the transgenic zebrafish *Tg (smyd1:m3ck)* exhibits a 2.16-fold higher ATP level and indeed maintains swimming behavior at temperatures of 13 degrees (52) vs. wildtype fish who cannot swim anymore, suggesting that energy availability may allow rapid changes in temperature to be tolerated. Second, following cold acclimatization, RNA transcripts involved in energy metabolism are upregulated, in particular those related to glycolysis (52, 53). Third, hypoxia treatment increases cold tolerance in zebrafish (54)—likely due to the resultant increased capability of zebrafish to undergo anaerobic respiration (55). These findings suggest that increased ATP availability and anaerobic respiration capabilities underlie cold acclimatization and tolerance. Indeed, lactate is found in the brains of cold-exposed zebrafish larvae (56), the end product of anaerobic respiration. Though larval zebrafish receive sufficient oxygen via diffusion to survive up to 6 dpf, active blood circulation increases oxygen uptake (57), and mutant larvae with bradycardia show signs of hypoxia and developmental retardation (58). Thus, the decrease in heartrate induced via cooling may be another factor in the shift of larval metabolism toward anaerobic respiration.

It is unclear whether the mechanism underlying the calcium surge is independent of the anesthetic effects, as it could potentially also underlie it. Should this not be the case, it still remains unclear whether this calcium wave could have been avoided by slowing the rate of cooling, or whether this would instead simply have delayed it. While it is possible that it may not have occurred, we believe that the cooling rate which was used here is in line with that of Collymore et al. (18), and can realistically be implemented in routine laboratory practice. Cooling rates used in the aquaculture industry are much slower [for example, 4°/h (59)], and thus incompatible with the workflows of most research laboratories carrying out experiments.

Tricaine and Cooling Decrease the Number of Stimulus-Modulated Neurons in the Hindbrain

Both tricaine and cooling led to a decrease in the number of stimulus-modulated neurons found in the hindbrain (see **Figures 5F,G**, **6C**). In our investigation of the pairwise cross-correlations of the activity of hindbrain neurons (**Figures 6E,F**), we observed a strong reduction of anti-correlations for both anesthesia treatments. These results suggest a loss of functional connectivity between the pretectum and hindbrain, as the remaining stimulus-tuned cells in the pretectum were no longer able to effect high levels of left-right alternating oculomotor activity in the hindbrain. Loss of functional connectivity is a defining characteristic of anesthesia (60–62), and a suggested mechanism behind loss of consciousness (63). Furthermore, both treatments appeared to increase the abundance of positive correlations, suggestive of a decrease in entropy, a reported characteristic of anesthesia. The use of alpha-bungarotoxin, a paralytic, has been shown to spare the anti-correlated-activity balance detected downstream in the spinal cord (64). Together, these results strongly suggest that tricaine and cooling act as anesthetics instead of simply suppressing startle and optokinetic responses via muscular paralysis.

Note that our results relate only to anesthesia in larvae and further research is needed to clarify the anesthetic effects of cooling in the adult zebrafish brain. Due to their larger size it is possible that it takes longer for the central brain to cool when compared to neurons in the spinal cord and peripheral nervous system. Crucially, while we found comparable onsets for tricaine and cooling anesthesia in larvae, a behavioral study in adults found that there are significant differences in the onset profiles between the two (18). In adults there was a delay of over 2 min between loss of equilibrium and loss of tactile response following tricaine exposure, while there was no such delay for gradual cooling.

CONCLUSION

Tricaine Is an Effective Anesthetic

The comparative effects of each treatment on the optokinetic response are summarized in **Figure 7**. The results of this study suggest that tricaine is an effective and potentially superior anesthetic in comparison to gradual cooling. A small number of motion-tuned neurons remains in sensory brain areas, and task-associated activity is almost completely abolished in the hindbrain. These results demonstrate that eye movements cease due to lack of behavioral drive rather than neuro-muscular paralysis, which confirms the anesthetic effect of tricaine. However, we found that even a 15-min exposure to tricaine could not completely silence motion-tuned neurons, so a higher dose of tricaine than the standard 168 mg/L concentration may be necessary to achieve higher levels of neural suppression. Of importance to the practical implementation of anesthesia, we did not observe any meaningful temporal discrepancy between the loss or recovery of reflexive eye movements and neural drive, suggesting that there is no lag between neuronal vs. behavioral onset and offset of the anesthetic effect of tricaine. Thus, observation of zebrafish movements during application of tricaine can serve as a reliable readout for the onset of anesthesia. We nonetheless caution readers that behavioral recovery took quite long (∼14 min), and it is possible that full recovery of neural activity occurs before the optokinetic behavioral recovery, during time periods we did not record.

Finally, gradual cooling should not be considered an appropriate alternative anesthetic agent until the long-term effects of the calcium surge have been fully investigated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Regierungspräsidium Tübingen in accordance with German federal law and Baden-Württemberg state law.

AUTHOR CONTRIBUTIONS

FDeh and AA: conceptualization. CL, FDeh, and AA: methodology and software. CL: formal analysis and data curation. CL, TB, FDeb, CS, and AA: investigation. CL and AA: writing—original draft preparation and visualization and supervision. CL, TB, FDeb, CS, FDeh, and AA: writing—review and editing. AA: project administration. FDeh and AA: funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.864573/full#supplementary-material

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Regulatory T Cell Modulation by *Lactobacillus rhamnosus* Improves Feather Damage in Chickens

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It is currently unclear whether potential probiotics such as lactic acid bacteria could affect behavioral problems in birds. To this end, we assessed whether a supplementation of *Lactobacillus rhamnosus* JB-1 can reduce stress-induced severe feather pecking (SFP), feather damage and fearfulness in adult birds kept for egg laying. In parallel, we assessed SFP genotypic and phenotypic-related immune responses and aromatic amino acid status linked to neurotransmitter production. Social stress aggravated plumage damage, while *L. rhamnosus* treatment improved the birds' feather cover in non-stressed birds, but did not impact fearfulness. Our data demonstrate the significant impact of *L. rhamnosus* supplementation on the immune system. *L. rhamnosus* supplementation induced immunosuppressive regulatory T cells and cytotoxic T cells in both the cecal tonsils and the spleen. Birds exhibiting the SFP phenotype possessed lower levels of cecal tonsils regulatory T cells, splenic T helper cells and a lower TRP:(PHE+TYR). Together, these results suggest that bacteria may have beneficial effects on the avian immune response and may be useful therapeutic adjuncts to counteract SFP and plumage damage, thus increasing animal health and welfare.

Keywords: psychoneuroimmunology, microbiota, social interaction, kynurenine, aromatic amino acids, laying hen

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INTRODUCTION

Poultry is the most extensively farmed land animal totalling approximately 26 billion birds worldwide in 2019 alone (1). Severe feather pecking (SFP) is a behavior commonly observed in birds kept for egg-laying, where female hens forcefully peck, remove and sometimes eat feathers of conspecifics (2). While some pecking is part of their natural behavior, SFP is a major behavioral problem as it causes feather cover damage and can develop into cannibalism (2). Birds rely on intact feather cover for thermoregulation/insulation and water-proofing (3), locomotion, and navigation of the environment (4, 5), and social communication (6). SFP is deleterious to the health and welfare of farmed birds. In addition, damage to the feather cover can lead to significant economic losses for commercial farms, for example through increased feed consumption to compensate for energy losses due to reduced feather cover (2). Flock mortality resulting from skin injuries and cannibalism events are also a non-negligible source of financial loss and can negatively impact consumers' trust and acceptance of poultry farming (2). Current farming practices aim to reduce consequences of the behavior rather than the behavior itself (7). Such practices, like beak trimming, are under increasing scrutiny because of animal welfare, ethical and societal concerns. Consequently, some commonly used procedures are being banned in multiple countries (7).

Despite decades of research, the cause of SFP is still unknown, attesting to the multifactorial nature of this behavior (2). Indeed, SFP can be influenced by physical and social environmental factors (8), as well as genetics (9), stress coping mechanisms (10), fearfulness (11), and neurobiology as determined by the monoaminergic systems (12, 13) or the immune system (14, 15). Of the multiple comorbidities associated with SFP, the involvement of the gut in the development of the behavior has gained attention. Descendants of White Leghorn pedigree lines that are bred for high or low SFP activity (9) are consistently reported to host distinct gut microbiota and short-chain fatty acid profiles (16–19). For instance, Birkl et al. (18) and van der Eijk et al. (19) found a lower abundance of *Lactobacillus* species in the cecal excreta of birds genetically selected for SFP behavior.

Lactobacilli are the predominant bacterial genus throughout the gastro-intestinal tract of chickens (20–23). Evidence suggests that they influence the gut-brain axis communication via an immune-mediated humoral pathway and a neural route (24–27). Lactobacilli are thought to impart beneficial effects on the stress response, the immune system, and stress-induced behavior in a diverse set of species (26, 28–31). For example, Lactobacillus supplementation increases T lymphocyte subpopulations in the gastro-intestinal tract of chicks, thereby impacting inflammatory processes (32). They also modulate the catabolic pathways of the aromatic amino acids (AAA), tryptophan (TRP) (33–35), phenylalanine (PHE), and tyrosine (TYR) (36). These AAAs are the precursors of kynurenine (KYN) and the monoaminergic neurotransmitters serotonin and dopamine, respectively (37, 38). The activities of the enzymes responsible for the TRP to KYN and PHE to TYR conversions are approximated by plasma KYN:TRP and PHE:TYR, respectively (39, 40). The TRP: (PHE+TYR) is a surrogate parameter reflecting the competition of TRP with other AAAs for uptake across the blood-brain-barrier (38).

Interestingly, the aforementioned physiological pathways influenced by lactobacilli are also interlinked with SFP. When considered together, these data suggest a gap in our understanding about the effects of the gut microbiome on SFP. We conducted a first study in adult hens selected for high SFP activity and showed that continuous oral intake of Lactobacillus rhamnosus improved feather cover, prevented stress-induced SFP behavior, changed regulatory T cell populations, and limited cecal microbiota dysbiosis (41). In a second study, the same L. rhamnosus strain was administered to chicks/pullets housed in large groups of low and high SFP genetic lines. Birds received the supplementation during early life to determine its efficacy as a preventative measure for SFP development under chronic stress. We found that L. rhamnosus caused a short-term increase in plasma TRP and TRP:(PHE+TYR), as well as an increase in all T lymphocytes of the spleen and cecal tonsils (42). L. rhamnosus is reported to modify gut motility within minutes of exposure ex vivo in mice (43) and chickens (44) and can reverse acute restraint stress-induced intestinal motility in mice (45). This demonstrates that Lactobacillus signaling can occur independently of colonization, alteration of the microbiome composition, or other longer-term adjustments (43). As such, it might be used as an immediate treatment against stress.

Consequently, the present study aimed to (1) evaluate the immediate impact of the oral administration of *L. rhamnosus* in response to stress by monitoring feather condition, fear behavior, and the immune and monoaminergic precursor responses in large mixed groups of low and high SFP laying hens, and (2) determine whether these physiological parameters are interrelated with the genetic background and the SFP phenotype of birds. To this end, we measured feather damage, SFP behavior, fear behavior, immunological markers, and actors (T-cells profiles, KYN:TRP ratio, and nitrite level) and markers of AAA metabolism (TRP, PHE, TYR, and their respective ratios).

MATERIALS AND METHODS

Ethical Statement

The experiment was approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #4113). To promote both refinement and reduction of bird numbers, the lines shared an experimental unit/pen (46).

Animals and Housing

Three pedigree lines of White Leghorn laying hens are maintained since 2015 at the University of Guelph Research Station (Guelph, Ontario, Canada). Yearly, this breeding flock is divergently selected for high (HFP) and low (LFP) severe feather pecking (SFP) activity or kept as unselected controls (UC) (9). Eggs were incubated, hatched in separate compartments per pedigree mother hen. At hatch, a total of 311 non-beak trimmed chicks were individually wing-tagged, and systemically allocated to 12 identical pens of 25 ± 2 birds each (8 ± 1 birds of each line; mixed lines per pen) in a windowless room. Each floor pen (1.6m^2) was littered with wood shavings and contained one round metal feeder (43 Ø cm), a drinker line (7 nipples), an A-frame perch (15 cm of perch/hen, 55 and 120 cm above the ground), and three nest boxes. The birds were able to hear other birds in neighboring pens, but visual contact was prevented by opaque PVC boards between the pens. Light was provided at 20 Lux from 05.00 h till 19.00 h and average daily temperature was 20 °C. Birds had *ad libitum* access to water and corn/wheat/soybean meal based feed (University of Guelph Research Station starter [0–6 weeks], grower [7–16 weeks] and layer [>17 weeks] mash diet) and housed under conventional management conditions at the research station. At 15 weeks of age (woa), one bird had to be put down due to cannibalism by conspecifics.

Lactobacillus rhamnosus Supplementation and Stress Treatment

An overview of the experimental timeline is presented in **Figure 1**. From 33 to 38 woa, six pens were systematically assigned to receive an oral supplementation with either *L.*

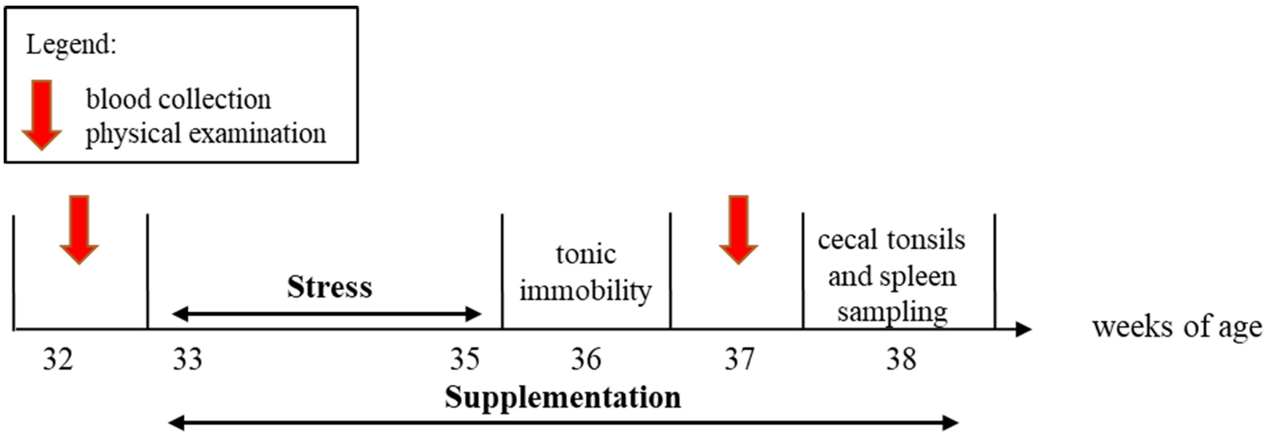

FIGURE 1 | Scheme of the experimental timeline. The *L. rhamnosus* or Placebo supplementation started at 33 weeks and lasted 6 weeks. The stress treatment spanned weeks 33–35. Physical examinations and blood collection were conducted at 32 and 37 weeks of age (woa). Tonic immobility was conducted at 36 woa. Spleen and cecal tonsils samplings were performed at 38 woa.

rhamnosus JB-1™ (Lacto, n = 6 pens, 157 birds) dissolved in drinking water or a placebo of drinking water (Placebo, n = 6 pens, 154 birds). *L. rhamnosus* JB-1™ was a gift from Alimentary Health Inc., Cork, Ireland to Paul Forsythe and Wolfgang Kunze, McMaster University.

Employing a supplementation method that can be easily adopted in a farm setting, birds were supplemented as a group within each pen. Supplementation was provided daily (Monday to Friday) between 9:00 h to 10:30 h. The Lacto treatment was prepared by dissolving 5 x 10^9 Colony Forming Units (CFU) of *L. rhamnosus* JB-1™ into 19 mL of warm drinking water per bird. To encourage birds to drink during the supplementation period, the drinker lines were raised to prevent water access for 1 h prior to supplementation. The Lacto or Placebo treatment were provided to the birds in their home pen using two round plastic 1L-drinkers (averaging 475 ± 38 mL per pen). Drinkers were monitored until they were voluntarily emptied (∼10 min), after which they were removed from the pens. Subsequently, the original drinker lines were lowered until the next round of supplementation.

At 33 woa, concomitant with the beginning of the supplementation treatment, three pens of each supplementation type were systematically assigned to a stress regimen in an attempt to induce SFP (13). The stress regimen lasted for 3 weeks (stress, $n = 156$ birds). The remaining three pens of Lacto and Placebo birds were left undisturbed (non-stress, $n = 155$ birds). Stressors were environmental (removal of perches and shavings, as well as blocking nest-boxes from Monday to Friday), and social (social disruption by mixing). Social disruption was repeated 3–4 times per week in the afternoon (14:00–17:00 h) for a total of 10 events. Stressed pens were split into two subgroups of three to four individuals and mixed with another subgroup from a different pen in the stress treatment assigned to the same supplement type (Lacto or Placebo). Upon mixing, birds were placed in a new, but identical pen to create a new environment for all birds. Wood shavings from the stressed pens were removed during the first mixing. This stress regimen was designed to mimic the unpredictable and repeated stressors that hens encounter in commercial farm settings (13, 47).

Behavioral Observations and Feather Damage Scoring

Prior to the experiment, birds were individually identified using continuously numbered silicone backpacks (8 x 6 x 0.5 cm) fastened onto the hens around the wings via two elastic straps secured to the backpacks with metal eyelets (48). Behavioral observations were conducted in the home pens via video recordings scheduled outside of the working hours of the farm staff to avoid any human bias. Cameras (Samsung SNO-5080R, IR, Samsung Techwin CO., Gyeongi-do Korea) were ceiling-mounted ahead of the trial to obtain a full view of each pen.

After determining the time windows during pilot observations, each pen was video recorded 10 min in the morning for a total of 100 min between 32 and 38 woa: 2 days at 32 woa as baseline, three days during the supplementation and stress treatments (35 woa), and 5 days post-stress treatment (36-38 woa). Behavioral recordings totaled 16 h of video and analysis was done by five trained blinded observers (Pearson's correlation of 0.88 for intra-observer reliability and 0.75 for inter-observer reliability) (42). All-occurrence sampling was used on all 311 birds to record the actor and recipient of SFP. SFP was defined as intent forceful peck(s) toward the feathers/body of conspecifics that may remove feathers or cause injury (2). All birds were individually examined for feather cover damage and bodyweight in weeks 32 and 37 by four blinded operators. Plumage damage to the neck, back and tail was assessed using a scoring scale adapted from Decina et al. with reported Kappa coefficients of 0.6-0.9 (49). The severity of plumage damage ranked from 0 (no or slight wear, nearly intact feathering) to 2 (at least one featherless area ≥ $2 Canadian coin, diameter: 28 mm). A fourth score (3) was added to account for the presence of at least one featherless area ≥ \$2 Canadian coin with fresh blood stains.

Tonic Immobility

A tonic immobility test was performed by four blinded operators over 3 days during week 36 as described by Jones and Faure (50) as a measure of fearfulness. Birds were individually removed from their home pen and tested in a nearby separate room. Birds were placed on their back in a U-shaped plastic cradle covered with a dark fabric. A standing operator then induced tonic immobility by gently restraining the bird for 15 s with one hand over the bird's breast and the other over the head. The induction was considered successful if the bird remained motionless for at least 10 s. After a successful induction, the operator sat and recorded the duration of tonic immobility, i.e., latency to self-righting. Birds were induced a maximum of three times, and the number of inductions needed was recorded. Birds still in tonic immobility after 5 min were given the maximum duration of 5 min.

Blood Sampling and Amino Acid Analysis

Blood samples were collected at 32 and 37 woa, from the wing vein using EDTA-coated vacutainer tubes (2 mL/hen). Individual birds were sampled within 1 h after their last meal on the same day of the week and at the same time of day (between 10:00 and 14:00 h) for both sampling points. Samples were gently inverted and stored on ice immediately after collection (maximum of 4 h). Plasma was separated by centrifugation at 4°C, 1,780 *g* for 15 min and stored at −80°C until further analysis.

The concentration of aromatic amino acids tryptophan (TRP), its derivative (kynurenine [KYN]), phenylalanine (PHE) and tyrosine (TYR), and nitrite were determined as reported previously (51). In brief, samples were analyzed via reversed-phase HPLC. The TRP, PHE and TYR concentrations were determined by monitoring their natural fluorescence (TRP: excitation [Ex] wavelength [λ] 286 nm, emission [Em] [λ] 366 nm; PHE, TYR: Ex λ 210 nm, Em λ 302 nm).

In mammals, the KYN to TRP ratio can be used to estimate TRP metabolism along the KYN axis, along which 90% of TRP not used for protein synthesis is catabolized. In humans, this ratio is used as an index of the IDO-1 activity if accompanied by an increase of immune activation markers such as neopterin (39). The PHE to TYR ratio may be used as a surrogate of phenylalanine 4-hydroxylase (PAH) activity, which converts PHE to TYR (40). TRP:(PHE+TYR) is a substitution for the commonly used ratio of TRP to large neutral amino acids. As described in Wurtman et al. (38), this ratio represents the competition of TRP with other amino acids for uptake across the blood-brain-barrier. However, it should be acknowledged that poultry physiology differs from mammals because of potential evolutionary variations (52, 53) and thus, the results should be approached with caution. As a surrogate marker of nitric oxide (NO) production, the stable NO metabolite nitrite was measured using a modified Griess assay (Merck KGaA, Darmstadt, Germany) (51).

Immune Phenotype

At week 38, 60 hens (five hens per line x supplementation type x stress treatment groups) were put down by cervical dislocation to determine T-cell populations as described in Mindus et al. (41). In brief, one cecal tonsil and the spleen were harvested from each bird within 3 min after death and kept in 5 mL of 5% fetal bovine serum (FBS) containing RPMI medium. Cells from both tissues were isolated, suspended, centrifuged, and counted. Viable spleen and cecal tonsils cells were diluted in fluorescence-activated cell sorting (FACS) buffer (PBS + 2% FBS) to a concentration of 10^6 cells/ml. Both splenocytes and cecal tonsil cells were stained for T-helper cells ($CD3^+CD4^+$ T cells), cytotoxic T lymphocytes ($CD3^+CD8^+$ T cells), and regulatory T cells ($CD4^+CD25^+$ T cells) markers using the same antibodies as in Mindus et al. (41). Data were acquired using FACSCelesta (Becton Dickinson, Oakville, ON, Canada) and analyzed by FlowJo (BD Bioscience, Ashland, OR, USA).

Statistical Analysis

FP frequencies were determined per individual per 10 min. Due to the low frequency of SFP, we focused on feather damage as a reliable indicator of the intensity of the behavior (54). The neck, back, and tail feather cover scores were used to assign a general plumage damage score (0–3; maximum score of the three body areas) for each bird at each sampling point. However, to further identify the physiological pathways linked to the behavior, we categorized birds as SFP peckers based on whether or not they had performed the behavior throughout the course of the experiment regardless of their genotype. Birds exhibiting at least one severe feather peck at 32, 35 or between 36–38 woa were categorized as severe peckers. Birds that performed 0 pecks were categorized as non-severe peckers.

The SAS software (version 9.4, SAS Institute, Cary NC) was used for all statistical computations. Unless specified, generalized linear mixed models (PROC GLIMMIX) were used to analyze the data. The assumptions of normally distributed residuals and homogeneity of variance were examined graphically with the use of QQ plots. Scatter plots of studentized residuals against predicted values and treatment values, and a Shapiro-Wilk test of normality were used to confirm the assumptions of the variance analysis. To detect possible outliers, studentized residuals outside a ± 3.4 envelope were used. Data was transformed where necessary. Least square (LS) means and standard errors on the data scale were recovered using the ilink option. Values are presented as LS means ± standard error, unless stated otherwise. Differences between means were compared pairwise using a Tukey-Kramer adjustment. Statistical significance was considered at $P < 0.05$.

Variances of plumage damage, bodyweight, tonic immobility duration and number of inductions to trigger tonic immobility, each T cell subset proportion, aromatic amino acid (AAA), KYN and ratios were partitioned into the fixed effect of supplementation, stress, line and their interaction with the best fitted distribution and their significance were tested through F-tests. When possible, the baseline values (collected at 32 woa) were used as covariates and the pens of the birds were designated as a random effect except for the tonic immobility outcomes

TABLE 1 | Percentage of birds receiving a given score for overall plumage damage within each treatment group.

Treatment	Class	Percentage of birds for each score (%)				OR	95% CI	*P*-value
		0	1	2	3			
Supplementation	Placebo	10	16	42	32	Ref	Ref	0.074
	Lacto	33	16	21	29	1.53	0.96–2.42	
Stress	NS	32	18	23	27	Ref	Ref	0.047
	S	11	15	39	35	0.63	0.39–0.99	
Supplementation x Stress	S-Lacto	13	10	30	47			<0.001
	S-Placebo	9	19	48	23			
	NS-Lacto	55	22	12	12			
	NS-Placebo	10	13	35	42			

The birds' plumage condition was assessed on the neck, tail, and back area (scale of 0 to 3; higher score indicating more severe damage) at 37 weeks of age. The maximum score from these areas was retained as the overall plumage condition. The odds ratio (OR) and 95% Confidence Interval (CI) were modeling the probability of a lower overall score (i.e., more intact feather cover). Birds received a supplementation treatment (water [Placebo] or L. rhamnosus [Lacto] supplementation, weeks 33–38) and stress treatment (non-stressed [NS] or stressed [S], weeks 33–35). Number of birds: S-Lacto = 79, S-Placebo = 77, NS-Lacto = 78, NS-Placebo = 77. Ref = Reference value.

(whereby observer within a day, the day and the pen were designated as random effects).

Finally, additional models were performed to identify whether physiological measurements were interrelated with the SFP phenotype. Variance of each T cell subset (obtained at 38 woa) and each AAA, their metabolites and ratios (obtained at 37 woa) was partitioned with the SFP phenotype (characterized from the behavior displayed from week 35 until week 37 [AAA variables determined from blood sampling] or week 38 [T cell subsets collected from tonsil/spleen sampling]) as a fixed effect and pen as a random effect.

RESULTS

Stress Aggravates Plumage Damage While *L. rhamnosus* Supplementation Improves Feather Cover Under Non-stressful Conditions

We assessed if the oral treatment with *L. rhamnosus* (Lacto) reduced stress-induced damage to the feather cover. We report that the stress treatment alone aggravated the severity of the overall feather damage ($F_{1,294} = 3.98$, $P < 0.05$, **Table 1**). Indeed, 74% of stressed birds had clear evidence of feather loss (score >2) compared to only 50% of non-stressed birds. Overall, the Lacto treatment favored the odds of less feather damage as only 51% of Lacto had clear evidence of feather loss (score >2) relative to 74% in the Placebo group ($F_{1,294} = 3.22$, P = 0.074, **Table 1**). However, there was a significant interaction between Lacto and stress treatments on the severity of plumage damage ($F_{1,294} = 27.30$, $P < 0.001$). Modeling the probability of birds having a lower score (i.e., more intact feather cover), we found that the Lacto treatment did significantly reduce plumage loss under non-stressed conditions. Indeed, the Lacto non-stressed birds were more likely to show more intact feather cover compared to the Placebo non-stressed (OR = 5.24, 95%CI = 2.62–10.46) and the Lacto-stressed birds (OR = 5.50, 95%CI = 2.72–11.08). This was reversed in the Placebo group, as Placebo stressed birds were more likely to present more intact feather cover than the Placebo non-stressed birds (OR = 2.1, 95%CI = 1.17–3.94). Finally, Lacto treatment did not improve feather cover under stressful condition as Lacto stressed birds were less likely to show intact feather cover (OR = 0.44, 95%CI = 0.24 - 0.82) than the Placebo stressed birds. With respect to the genetic lines, birds from the HFP line tended to be more likely to have more intact feather cover than the birds from the LFP line (Means ± SD, HFP:1.5 ± 1.08 vs. LFP 2.0 ± 0.99; OR = 1.74, 95%CI = 1.01–2.99).

L. rhamnosus Treatment Can Buffer Against Bodyweight Loss but Does Not Affect Fearfulness

We determined the effects of Lacto treatment and stress on bodyweight and its implications for fear behavior as measured through the duration of immobility and number of inductions in a tonic immobility test. We found that Lacto treatment prevented stress-induced bodyweight loss. Indeed, there was a significant interaction between Lacto and stress treatment in determining bodyweight ($F_{1,296} = 6.11$, $P = 0.014$). Placebo non-stressed birds were 3% heavier compared to the Placebo stressed birds (Placebo non-stressed: 1.77 ± 0.008 vs. Placebo stressed 1.72 ± 0.008 kg, $P < 0.001$). We observed no significant difference in bodyweight between stressed and non-stressed birds in the Lacto birds (Lacto non-stressed: 1.75 ± 0.008 vs. Lacto stressed 1.74 ± 0.008 kg, $P > 0.05$).

The number of inductions required to enter tonic immobility and its duration were not impacted by the Lacto treatment, stress or by their interaction ($P > 0.05$, **Supplementary Table 1**). However, HFP birds had significantly shorter tonic immobility durations (HFP: 60 ± 9.5 s vs. LFP: 89 ± 13.8 s, $F_{2,278} = 3.69$, $P = 0.026$) and necessitated more inductions for tonic immobility (HFP: 1.7 ± 1.01 vs. LFP: 1.4 ± 0.77, $F_{2,283} = 0.03$, $P = 0.006$) than LFP birds, while UC birds showed a more intermediate response (duration: 72 ± 11.2 s, inductions: 1.6 ± 0.89).

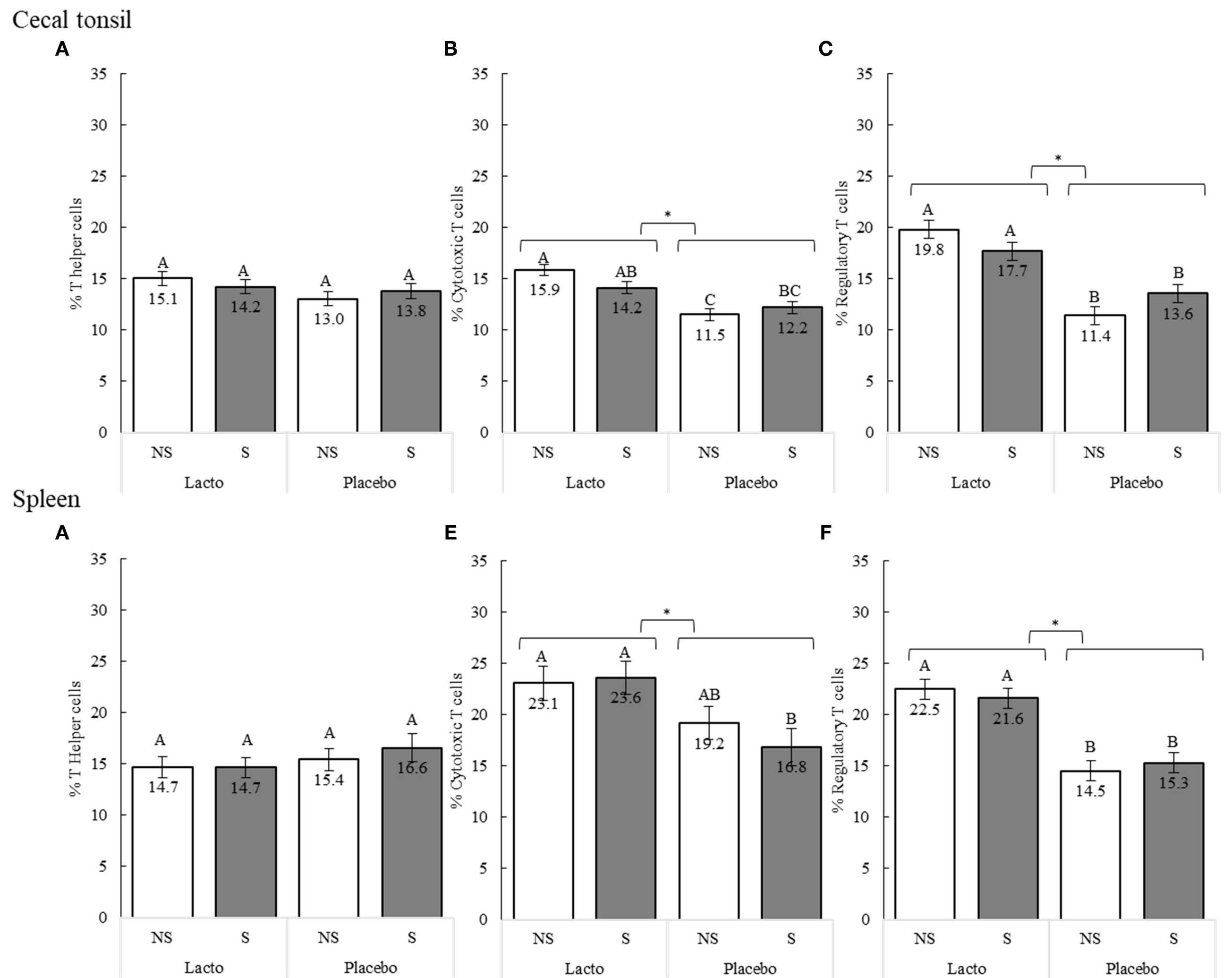

FIGURE 2 | Proportions of T cell sub-populations in the cecal tonsils **(A–C)** and spleen **(D–F)** of 38-weeks old birds after 5 weeks of supplementation (*L. rhamnosus* [Lacto] or water [Placebo] supplementation, 33–38 weeks of age) and 3 weeks of stress treatment (non-stressed [NS] or stressed [S], 33–35 weeks of age). Sub-populations were identified using the following combinations of cell surface markers: T helper cells = $CD3^+CD4^+$; cytotoxic T cells = $CD3^+CD8^+$; T regulatory cells = $CD4^+CD25^+$ (n of birds: S-Placebo = 15, NS-Placebo = 15, S-Lacto = 15, NS-Lacto = 15). Different superscript letters indicate statistically significant differences between specific supplement and stress treatment comparisons of the interaction, and * indicates statistically significant differences of the supplementation treatment as a main effect ($P < 0.05$).

Oral Treatment of *L. rhamnosus* Induces a Strong Regulatory T Cell Response

In poultry species, chronic stress decreases the proportion of peripheral blood lymphocytes (55), an outcome that can be countered by lactobacilli (32, 41, 42). We assessed the capacity of L. rhamnosus treatment to stimulate T helper cells ($CD3^+CD4^+$ T lymphocytes), cytotoxic T cells ($CD3^+CD8^+$ T lymphocytes), and regulatory T (Treg) cells ($CD3^+CD4^+CD25^+$ T lymphocytes) in the spleen and cecal tonsils of laying hens in response to a stress treatment induced between 33 and 35 woa (**Figure 2**).

The Lacto and stress treatments interacted to determine the proportion of the cytotoxic T cells ($F_{1,47} = 4.30$, P = 0.044, **Figure 2B**) and Treg cells ($F_{1,47} = 5.85$, P = 0.020, **Figure 2C**) in the cecal tonsils. While the Lacto supplementation generally increased the proportions of these cells compared to the Placebo, the difference was larger in non-stressed groups. In contrast, a significant increase of splenic cytotoxic T cells (**Figure 2E**) was only observed in the stressed groups (P = 0.009), while no significant difference was found between the non-stressed groups (P = 0.222). The stress treatment alone did not influence T cell proportions ($P > 0.05$). However, overall, Lacto treatment increased the proportion of Treg cells in the cecal tonsils ($F_{1,47} = 51.40$, $P < 0.001$) and spleen ($F_{1,47} = 53.91$, $P < 0.001$), as well as the proportion of cytotoxic T cells in both tissues (cecal tonsil, $F_{1,47} = 29.26$, $P < 0.001$; spleen, $F_{1,47} = 13.94$, $P < 0.001$) compared to Placebo birds (**Figure 2**). The proportion of T helper cells

TABLE 2 | Least squares means (± standard error) of the proportions of T cell sub-populations in the spleen and cecal tonsils in 38 weeks old birds according to their genetic line (UC: unselected control, LFP: low feather pecking line, HFP: high feather pecking line).

	Lacto			Placebo			
	UC (n = 10)	LFP (n = 10)	HFP (n = 10)	UC (n = 10)	LFP (n = 10)	HFP (n = 10)	Supplementation x line F- Statistic, P-value
Cecal tonsils							
T helper cell	15.2 ± 0.82	13.9 ± 0.83	14.9 ± 0.83	12.7 ± 0.83	14.4 ± 0.85	13.2 ± 0.85	$F_{2,47}$ = 2.00, P = 0.147
Cytotoxic T cell	16.2 ± 0.70 ^{a}	13.5 ± 0.70 ^{ab}	15.2 ± 0.70 ^{a}	11.1 ± 0.70 ^{b}	13.4 ± 0.69 ^{ab}	11.1 ± 0.69 ^{b}	**$F_{2,47}$ = 6.75, P = 0.003**
Regulatory T cell	21.4 ± 1.08 ^{a}	17.5 ± 1.08 ^{ab}	17.5 ± 1.08 ^{ab}	11.2 ± 1.08 ^{c}	14.8 ± 1.08 ^{bc}	11.5 ± 1.08 ^{c}	**$F_{2,47}$ = 6.12, P = 0.004**
Spleen							
T helper cell	2.7 ± 0.08	2.7 ± 0.08	2.6 ± 0.08	2.8 ± 0.08	2.8 ± 0.09	2.7 ± 0.09	$F_{2,47}$ = 0.29, P = 0.751
Cytotoxic T cell	23.4 ± 1.9 ^{ab}	25.0 ± 1.9 ^{a}	21.6 ± 1.9 ^{ab}	18.1 ± 1.9 ^{ab}	17.7 ± 2.01 ^{b}	18.2 ± 2.03 ^{ab}	$F_{2,47}$ = 0.65, P = 0.526
Regulatory T cell	27.5 ± 1.19 ^{a}	18.6 ± 1.19 ^{b}	20.1 ± 1.19 ^{b}	13.5 ± 1.19 ^{c}	16.1 ± 1.19 ^{bc}	15.1 ± 1.19 ^{bc}	**$F_{2,47}$ = 12.89, P < 0.001**

	Stress			Non-stress			
	UC (n = 10)	LFP (n = 10)	HFP (n = 10)	UC (n = 10)	LFP (n = 10)	HFP (n = 10)	Stress x line F- Statistic, P-value
Cecal tonsils							
T helper cell	12.8 ±0.82	14.9 ± 0.85	14.4 ± 0.85	15.1 ± 0.83	13.4 ± 0.83	13.6 ± 0.83	$F_{2,47}$ = 3.05, P = 0.057
Cytotoxic T cell	12.8 ± 0.70	14.8 ± 0.69	11.9 ± 0.68	14.5 ± 0.69	12.1 ± 0.69	14.3 ± 0.69	**$F_{2,47}$ = 7.39, P < 0.001**
Regulatory T cell	15.2 ± 1.08 ^{ab}	18.3 ± 1.08 ^{a}	13.4 ± 1.08 ^{b}	17.4 ± 1.08 ^{ab}	14.0 ± 1.08 ^{ab}	15.5 ± 1.08 ^{ab}	**$F_{2,47}$ = 5.84, P = 0.005**
Spleen							
T helper cell	2.8 ± 0.08	2.8 ± 0.09	2.7 ± 0.09	2.7 ± 0.08	2.8 ± 0.08	2.6 ± 0.08	$F_{2,47}$ = 0.21, P = 0.812
Cytotoxic T cell	19.1 ± 1.90	22.9 ± 2.01	18.6 ± 2.02	22.5 ± 1.93	19.7 ± 1.93	21.2 ± 1.91	$F_{2,47}$ = 2.16, P = 0.127
Regulatory T cell	18.9 ± 1.19 ^{abc}	20.0 ± 1.19 ^{ab}	16.4 ± 1.19 ^{bc}	22.0 ± 1.19 ^{a}	14.7 ± 1.19 ^{c}	18.8 ± 1.19 ^{abc}	**$F_{2,47}$ = 7.50, P = 0.002**

Birds underwent 5 weeks of supplementation (L. rhamnosus [Lacto] or water [Placebo] supplementation, 33–38 weeks of age) and 3 weeks of stress treatment (33–35 weeks of age). Sub-populations were identified using the following combinations of cell surface markers: T helper cells = $CD3^{+}CD4^{+}$; cytotoxic T cells = $CD3^{+}CD8^{+}$; T regulatory cells = $CD4^{+}CD25^{+}$. Different superscript letters indicate statistically significant different comparisons within the interaction in each row at P < 0.05.
F-Statistics and P-values of the supplementation x line or stress x line interaction; interaction terms significant at P < 0.05 are bolded.

was not affected by the Lacto and stress treatment, or their interaction (P > 0.05).

To better understand the physiological pathways underlying SFP behavior, the interrelatedness of the immune response with the genetic lines and the SFP phenotype from week 35 to week 38 (tissue collection) were evaluated. We found that lines reacted differently to the Lacto and stress treatments (**Table 2**). Indeed, Lacto treatment increased the proportion of Treg cells in the HFP and UC birds compared to the Placebo in the tonsils ($F_{2,47}$ = 6.12, P < 0.01), and to a lesser extent the spleen ($F_{2,47}$ = 12.89, P < 0.01). No difference in Treg cells was observed in the LFP birds following Lacto treatment (**Table 2**). Inversely, Lacto birds had a higher proportion of splenic cytotoxic T cells in LFP birds compared Placebo birds (P = 0.049), while no difference was observed in the other lines (P>0.05, **Table 2**). Stress increased the proportion of splenic Treg cells in the LFP line compared to non-stressed birds (P = 0.035). No difference was observed in the other lines (P > 0.05, **Table 2**). Stressed HFP birds had fewer tonsil Treg cells than stressed LFP birds (P = 0.027), while no difference was observed in the non-stress birds (P = 0.924, **Table 2**).

We found that the genetic line alone impacts the proportion of splenic Treg cells ($F_{2,47}$ = 4.24, P = 0.020, **Supplementary Table 2**). The UC line had a significantly higher proportion of Treg cells than the HFP (P < 0.05) and LFP (P = 0.032) lines; but there were no differences between the HFP and LFP birds (P = 0.981). Other proportions of T cells were similar between the genetic lines (P > 0.05, **Supplementary Table 2**). Phenotypical severe feather peckers (i.e., birds who performed at least one severe feather peck between 35-38 woa) were retrospectively identified and mostly came from the HFP (52%), UC (38%), and lastly LFP (10%) line as expected. Phenotypical severe feather peckers had reduced levels of Treg cells in the tonsils ($F_{1,53}$ = 4.06, P = 0.049) and splenic T helper cells ($F_{1,53}$ = 4.07, P = 0.049) compared to non-peckers (**Supplementary Table 2**).

SFP Phenotype Is Associated With Lower TRP:(PHE+TYR) and Elevated TYR Levels

We investigated the impact of Lacto and stress treatments on the concentrations of aromatic amino acids (AAA), the TRP metabolite KYN, ratios, and nitrite at 37 weeks of age.

L. rhamnosus supplementation, stressors, and their interaction did not significantly change peripheral plasma levels of TRP, PHE, TYR, KYN, and their relevant ratios. Furthermore, the TRP:(PHE+TYR) and nitrite concentrations were similar between groups (**Supplementary Table 3**).

We examined whether the AAA response was related with the genetic lines and SFP phenotype displayed from week 35 until week 37 (blood collection). Genetic line x stress interactions showed that LFP birds differ in their stress responses compared to birds from other lines (**Supplementary Table 4**). Indeed, in LFP birds, stress increased KYN concentrations (stressed: 0.38 ± 0.022 μM vs. non-stressed: 0.29 ± 0.021 μM, $F_{2,277} = 4.69$, $P < 0.01$) and KYN:TRP (stressed: 4.3 ± 0.25 μmol/mmol vs. non-stressed: 3.3 ± 0.25 μmol/mmol, $F_{2,277} = 4.68$, $P = 0.01$) levels while no change was observed in the stressed vs. non-stressed birds of the HFP and UC lines.

Overall, peripheral plasma levels of TRP, PHE, TYR, KYN, their relevant ratios, and the nitrite concentrations were similar between genetic lines ($P > 0.05$, **Supplementary Table 5**). Nevertheless, we report that phenotypic severe feather peckers had significantly lower TRP:(PHE+TYR) than birds that were not severe feather peckers (severe feather peckers: 0.35 ± 0.010 μmol/μmol vs. not severe feather peckers: 0.38 ± 0.007 μmol/μmol, $F_{1,292} = 9.70$, $P = 0.002$). Peckers also tended to have lower peripheral plasma TYR concentrations (severe feather peckers: 128 ± 3.5 μM vs. not severe feather peckers: 122 ± 2.2 μM, $F_{1,288} = 3.72$, $P = 0.055$).

DISCUSSION

The aim of the present study was to determine whether oral supplementation with a single *L. rhamnosus* strain can act as an immediate measure to reduce stress-induced severe feather pecking (SFP) and associated physiological changes. To this end, we assessed the feather cover, tonic immobility duration and number of inductions, T cells profiles, aromatic amino acids metabolism, along the kynurenine (KYN) and dopaminergic pathways quantified by plasma tryptophan (TRP), phenylalanine (PHE) and tyrosine (TYR) concentrations in laying hens following 5 weeks of supplementation (33–38 weeks of age [woa]) and a concomitant three-week stress regimen (33–35 woa). Three genetic lines of birds housed in mixed groups were used in this study: high feather pecking (HFP), low feather pecking (LFP) and unselected control (UC). We also analyzed the link between genotypic and phenotypic SFP behavior and physiological parameters. We report that stress aggravates the severity of damage to the feather cover while *L. rhamnosus* supplementation mitigated the feather damage in non-stressed conditions. Surprisingly, *L. rhamnosus* supplementation did not mitigate the damage to feather cover in stressed conditions. Furthermore, the *L. rhamnosus* supplementation induced immunosuppressive regulatory T cells (Treg) and cytotoxic T cells in both the cecal tonsils and the spleen. Birds exhibiting the SFP phenotype displayed lower levels of tonsil Treg and splenic T helper cells as well as a lower TRP:(PHE+TYR).

Considering the biological importance of feather cover (2), it is important to note that stress alone deteriorated the overall feather cover. Nearly 75% of stressed birds had clear evidence of feather loss, reflecting previous findings (13, 41). Most importantly, we found that the Lacto supplement tended to decrease the severity of the feather damage (**Table 1**), however, this effect was only found in non-stressed birds. Taken together, these results agree with mammalian studies in which *Lactobacillus* bacteria are known to have a positive influence in healthy, non-stressed individuals (29, 56, 57). Importantly, this finding was not replicated in the stressed birds where Lacto stressed birds actually had more feather damage than Lacto non-stressed birds (**Table 1**). Potentially, the current stress regimen overrode the potential beneficial effects of the supplementation in this study. Previous research using a more varied stress regimen (e.g., social disruption, shavings replacement, individual and group restraint, blocking nest boxes and perches in random order) showed that ingestion of *L. rhamnosus* positively modulates chronic stress-induced feather damage when continuously administered before, during and after stress to adult HFP birds (41). The fact that different results were observed in the current study when Lacto was supplemented only during and after stress, could highlight the importance of relative timing of probiotic supplementation. Interestingly, a recent meta-analysis showed that the order in which the *Lactobacillus*-based probiotics and stress treatments are applied does not change the effect of the probiotic in rodents (31). However, Liu et al. (58), showed that *L. rhamnosus* supplementation administered only post-social stress increased the persistence of both aggressor avoidance and reduced sociability in stressed mice. Thus, although the present variables (pecking behavior and feather damage) are distinct from Liu et al. (58), (sociability and avoidance), the effect of *L. rhamnosus* supplement could vary depending on whether it was administered before, during or after stress exposure. That said, the Lacto treatment did appear to be protective against stress-induced weight loss with no difference in body weight between stressed and non-stressed Lacto birds, while the stressed-placebo birds weighed 3% less than their non-stressed counterparts. Regardless, further work on the potential beneficial effects of *L. rhamnosus* supplementation, even under non-stressful conditions, should not be overlooked as intact feather cover has both biological and economic benefits, e.g., by improving bird welfare and reducing farm feed costs (2).

The proportion of T-cell subsets, except for the splenic T helper and Treg cells, were 2 to 3-fold lower than previously observed in younger birds (10 and 28 weeks) of similar lines (15, 41), which is most likely an age-related effect (59, 60). However, the dysregulation of T cell (without subset specification) proliferation and activation has been suggested to be the initial cause of the FP phenotype possibly via cholinergic signaling (61). Interestingly, we also report that tonsil Treg cells in severe feather peckers are 22% lower than in non-peckers (**Supplementary Table 2**). Tregs are a population of immunosuppressive $CD4^{+}$ T lymphocytes involved in maintaining immune tolerance to self-antigens and preventing autoimmune/autoinflammatory disease (62). As such, Tregs help suppress inflammatory responses (63). Chicken Treg cells have suppressive properties similar to that of mammalian Treg cells (64) and depleted Treg populations have been associated with negative changes in mood and behavior in animal models (65–68). Our results support the idea that SFP could be an immune-related behavioral response, and more specifically, that Treg cells could play a role in determining SFP behavior.

We further report that *L. rhamnosus* supplementation had a strong immunomodulatory effect in laying hens, whereby it increased Treg cells and cytotoxic T cells in the spleen and cecal tonsils compared to birds receiving the Placebo. These results are consistent with previous mammalian (69–73) and avian studies (41, 42, 74). Apart from increased Treg cells, *L. rhamnosus* treatment also had anti-depressive and anxiolytic effects in mice (26, 73), and these effects were mediated through Treg cells (75). This implies a link between the immune response and behavior in mammals. To assess differences in reactivity behavior, we conducted a tonic immobility test, a well validated standardized test of fearfulness in chickens (76). We found that SFP and tonic immobility outcomes were correlated to the proportion of Treg cells in Lacto birds (data not shown), even if Lacto and stress treatment did not directly impact birds' fear responses. Indeed, severe feather peckers supplemented with Lacto had increased proportions of Treg cells in the tonsils and reduced level of SFP compared to the Placebo birds (data not shown). Additionally, low levels of splenic Tregs were associated with longer tonic immobility duration ($r = -0.39$, $P = 0.034$) and fewer induction of tonic immobility ($r = 0.43$, $P = 0.019$), suggesting a more fearful state. Similarly, a lower proportion of tonsil Tregs was associated with fewer tonic immobility inductions ($r = 0.43$, $P = 0.017$). These correlations were not significant in Placebo birds. Thus, we propose that *L. rhamnosus* oral supplementation positively modulates the immune system through Treg cell induction and that *L. rhamnosus* supplementation is positively linked to social interactions such as SFP behaviors and indirectly to fear in laying hens, mirroring work in mammalian models. Immunosuppressive Treg cells may then play an essential role in mediating the avian gut-brain axis signaling. It would be of further interest to investigate this hypothesis and test if the mechanism of T cell induction via *L. rhamnosus* supplementation is similar to mammals. In mice, *L. rhamnosus* is taken up by dendritic cells in the Peyer's patches, which then induce Treg cell production (71), a process that is vagus nerve-dependant (26).

Severe feather peckers had significantly lower peripheral TRP:(PHE+TYR) and higher TYR concentration than birds that did not express SFP (**Supplementary Table 5**). Previous studies unambiguously identified monoamine signaling as a key component in SFP behavior, mostly due to low central serotonin and dopamine turnover at a young age (12, 77, 78). We focused on AAA as these are the precursor metabolites for neurotransmitters of the serotonergic and catecholaminergic systems. We estimated their concentrations in the blood, and besides catabolism also transports affects the CNS availability, thus these both processes were taken into account by calculating relevant ratios. AAA concentrations were interesting to get first insights on the potential modulation of the crosstalk between the nutritional status and the serotonergic and catecholaminergic neurotransmitter biosynthesis upon *L. rhamnosus* supplementation and/or stress treatment. However, we found no variation of peripheral plasma concentrations of TRP, PHE, TYR, and KYN, their relevant ratios, and nitrite at 37 woa in response to 3 weeks of Lacto supplementation and stress. Previously, we demonstrated that early-age consumption of *L. rhamnosus* for 8 weeks led to a short-term increase of peripheral TRP concentrations and TRP:(PHE+TYR) in pullets (42). Amino acid and peptide absorption in birds (79) is similar to that in mammals (80). Still, during the first few weeks following hatch, the intestinal tract of birds grows rapidly (80). This may explain why no difference was observed in mature birds who were supplemented after the microbiome would be established in the current study (supplementation 33-38 woa) and previously reported (supplementation 19-28 woa) (41) compared to the study with pullets (supplemented 0-9 woa) (42). While *L. rhamnosus* has been shown to impact specific neurotransmitters-like GABA receptor expression (26), our results suggest that the effect on AAAs and related neurotransmitters pathways might be minimal, short-term or it suggests that the five weeks of *L. rhamnosus* supplementation may not be sufficiently long to impact monoamines precursors. However, it needs to be mentioned that there are still many unexplained variables, also regarding basic avian metabolism. For example, only IDO-2, which is less efficient in breaking down TRP along the KYN pathway than IDO-1, has been detected in birds and the regulation of the avian enzyme is still poorly understood in terms of modulation of activity by nutritional or potentially also stress or immunological factors (53).

It is noteworthy that the overall expression of SFP was scarce with an average frequency of 0.078 ± 0.555 pecks/bird/10 min across all birds between 35 and 38 woa. The observed level of SFP was approximately 7 fold lower than frequencies observed in 16-weeks old pullets (13) and 28–29 weeks old birds (11) of the same genetic HFP line. Despite pilot testing, it cannot be ruled out that the time window of behavior recording may not accurately reflect the true behavioral outcomes which may have impacted the results. Direct recording of behavior in large groups of birds is difficult and time consuming to perform, and scoring of feather cover provide a reliable estimation of the intensity of the behavior (54). Moreover, it can be argued that in commercial farms, feather damage, rather than the behavior itself, is of interest due to its aforementioned welfare and economic consequences. For this reason, we mainly focused on the feather damage outcome in the current study. However, because the purpose of our study was also to identify the physiological pathways linked to the behavior, we did record SFP during the experiment which allowed us to retrospectively determine bird phenotypes.

We additionally investigated the differences between the genetic lines. The present experiment mixed the HFP, LFP and UC genetic lines equally within the housing pens. This was done to mimic commercial conditions, but could have influenced the occurrence of feather damage and performance of SFP as LFP birds could be at higher risk of being victims or have learned to perform this behavior from the HFP birds (81) compared to if they were housed in groups according to their genetic line. This mixed housing of genetic lines could potentially also explain why the levels of feather damage were relatively high even in the non-stressed group. Additionally, this may partly explain the variability in the findings compared to Mindus et al. (41), who used only HFP birds. Indeed, differences in stress response (8, 10, 82), as well as in the reactivity of the immune system between various genetic lines (8, 15), are well documented. Similar to van der Eijk et al. (11), we found that HFP birds spent less time in and needed more inductions to be in tonic immobility, suggesting that they were less fearful than LFP birds (50, 76). Interestingly, LFP birds had more damaged feather cover than HFP birds, which may have enhanced their fearfulness. While we did not observe differences in fear response or feather damage to the Lacto or stress treatments between the genetic lines, we did observe that LFP birds stand out in their stress response in other physiological responses. Indeed, stress usually suppresses the avian immune system (55, 83, 84), an effect that was observed in the HFP and UC lines where, overall, all T cells proportions were lower in the stress groups (**Table 2**). On the contrary, the stress treatment increased the proportion of all T cells in the LFP line. Nevertheless, this increase was only significantly different for the splenic Treg cells (**Table 2**). Similarly, stress increased peripheral KYN concentrations and KYN:TRP in the LFP line, but not in the HFP and UC lines (**Supplementary Table 4**). These results may suggest that the LFP birds may be less sensitive to stress or that their stress-sensitive physiological pathways have a different regulation (85). These differences in stress response between the genetic lines were not, however, reflected in the SFP phenotype displayed from week 35 until blood/tissue collection at week 37–38. Thus, it is unclear whether they might play a role in SFP behavior. The causation of these differences should be further investigated to better understand the relationship between these physiological pathways and feather pecking behavior.

Mindus et al. (41) showed that individual Lacto supplementation of birds at 5×10^9 CFU/bird prevented stress-induced SFP. In the present study, birds were treated with the *L. rhamnosus* supplement as a group to mimic commercial farm conditions which precludes individual administration of treatments as thousands of birds comprise a typical flock. We introduced the equivalent of 5×10^9 CFU of *L. rhamnosus* per bird *via* two 1L-round drinkers and ensured that the full volume was consumed. This strategy prevented measuring individual consumption. Regardless of the individual dosage received, we observed strong and immediate immunomodulatory effects. Nevertheless, as *Lactobacillus* bacteria show some dose-dependent response in mammals (86–88), it is possible that this dosage was not sufficient to alter SFP behavior in the genetic lines of birds used in the present study. Similarly, individual feed consumption was not measured. Amino acids concentrations are largely controlled by total dietary intake, and thus, variations in feed consumption may have impacted the observed results.

CONCLUSION

To study the impact of probiotic bacteria as an immediate measure against stress-induced feather damage, we supplemented adult laying hens with a daily dosage of *L. rhamnosus*, while following a validated stress regimen. We also investigated whether the immune and aromatic amino acids responses differed between the genetic lines and were interrelated with the severe feather-pecking phenotype itself to better understand the underlying physiological pathways of this behavior. Three weeks of stress treatment aggravated the severity of plumage damage. *L. rhamnosus* supplementation improved the birds' feather cover under non-stressful conditions; however, considering our previous study (41) *L. rhamnosus* supplementation needs to be provided ahead of stressful conditions. The severe feather-pecking phenotype was linked to lower proportions of regulatory T cells and lower TRP:(PHE+TYR). *L. rhamnosus* supplementation increased regulatory and cytotoxic T cells in the spleen and cecal tonsils, which were also correlated to birds' fear responses during tonic immobility. Thus, *L. rhamnosus* supplementation may modulate SFP and fearfulness via regulatory T cells induction. Our findings help elucidate biological mechanisms that are associated with SFP behavior and the pathways through which *L. rhamnosus* supplementation may mitigate behavior. These results pave the way for a better understanding of how individualized, microbial interventions can help reduce feather damage in commercial farms, and thus, improve the welfare of millions of domestic birds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Guelph Animal Care Committee (Animal Utilization Protocol #4113).

AUTHOR CONTRIBUTIONS

CM, NS, WK, PF, and AH-M: conceived and designed the experiment. WK, PF, and AH-M: secured the funding for the experiment. CM and NS: performed the experiment and collected the data at the research station. DF, JG, and MM: processed the samples. CM: conducted the statistical analysis and wrote the original draft. CM, NS, DF, JG, JK, WK, MM, AS, PF, and AH-M: reviewed and approved the final manuscript before submission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.855261/full#supplementary-material

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Space, time, and context drive anticipatory behavior: Considerations for understanding the behavior of animals in human care

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Animal-based measures reflecting the welfare state of individuals are critical for ensuring the well-being of animals under human care. Anticipatory behavior is one potential animal-based measure that has gained traction in recent years, as it is theorized to relate to animals' reward sensitivity. It is of particular interest as an assessment for animals living under human care, as the predictability of the captive environment lends itself to the development of this class of behaviors. Animals are likely to exhibit anticipation in locations related to the anticipated event, often in temporally predictable time frames, and before specific contexts they experience in their day-to-day management. In this sense and under certain circumstances, anticipatory behaviors are likely to drive observed behavioral or space use patterns of animals under human care. Drawing conclusions from such data without identifying anticipation may result in misleading conclusions. Here we discuss how space, time, and context are related to patterns of anticipatory behaviors in animals under human care, how unidentified anticipation may alter conclusions regarding animal behavior or welfare under certain circumstances.

KEYWORDS
animal welfare, welfare indicator, reward sensitivity, zoo animal, welfare assessment

Introduction

Anticipatory behavior is a common phenomenon, documented in numerous species (1–9), in facilities where animals are cared for by humans (10). Briefly, anticipatory behavior is a suite of behaviors exhibited by animals during the appetitive phase (i.e., the searching phase of a behavioral sequence), aimed at the acquisition of a resource (11, 12). Readily observable anticipation is likely to develop under conditions where the availability of resources is predictable, either due to timing or cues in the environment (4, 13).

The past decade has seen a proliferation of animal welfare focused studies that assess anticipatory behavior to gain an understanding of animals' emotional states (14–17). This growth in research is evident in zoos and aquariums (hereafter zoos) where accreditation standards globally increasingly require assessing the welfare of all species living in zoological institutions (18–21). Anticipatory behavior provides a unique opportunity to study psychological states of animals and the factors influencing them with little manipulation. Indeed, in comparison to other approaches thought to assess animals' own reflections of their underlying psychological state, such as cognitive bias assessments, observations of anticipatory behavior rely on minimal intervention (9, 22, 23).

The manifestation of anticipatory behavior is greatly affected by many factors. Animals under human care generally receive primary reinforcing components of their care either on fixed schedules, following reliable or semi-reliable cues associated with them, or some combination of these. Thus, animals may anticipate based on the timing of events or the timing and sensory modality of the cues associated with these events (4, 24–26). The physical structure of the environment in which the behavior occurs can influence this behavior, as positive opportunities commonly occur in the same location(s) in the animals' space, such as feeding in a regular location or training sessions occurring where staff can access the animal (9, 23, 25). Additionally, the contexts in which the events occur can influence the manifestation of anticipatory behaviors (27, 28). Context itself can be multifaceted with variations in season, social situation, and cyclically hormonally-driven motivations (28–31).

Anticipatory behavior can be observed in animals that live in either captive or wild settings. Liberal interpretations of the behavior include all forms of behaviors associated with appetitive responses aimed at the acquisition of any perceived need (11, 32, 33). More conservative interpretations include responses tied to a clearly discernible (by the observer) cue(s) or to an observable pattern in the timing of events (11, 32, 33). Animals in wild settings thus express anticipatory behavior in a variety of ways and the general class of behavior is a core component of an ecologically relevant behavioral time budget.

Early studies labeled anticipatory behavior as 'food anticipatory activity' and demonstrated this behavior can become quite pronounced when animals rely on humans for scheduled caretaking (6, 13, 34, 35). These studies also occurred in laboratory settings where the factors that can shape the behavior were greatly simplified. Zoos provide more complex environments than those typically afforded lab animals, but are also subject to similar issues of scheduled care events, which can foster the development of anticipatory behavior (10). More recently, zoos have undergone a strong shift toward a focus on animal welfare (36) and emulating environments more in line with those the animals evolved in (37); nevertheless, much of the described behavior in zoo animals, the manner by which they utilize space, engage in daily rhythms, and even interact with conspecifics is shaped by patterns of anticipatory behavior. This may be particularly true in older descriptions that predate a focus on providing animals enriched environments (38) and longer lasting opportunities to be engaged in their environment (39). Anticipatory patterns across species appear to have specific relationships to the environmental contexts, timing of daily care events, and the spaces animals experience in their daily lives. Thus, unidentified anticipatory patterns have the potential to alter conclusions drawn from behavioral observations (40).

Here, we review how anticipation is expressed across space, time, and under different contexts. We discuss potential challenges of drawing conclusions from behavioral data collected from animals exhibiting anticipatory behaviors, and potential methods to identify or account for anticipation within existing datasets.

Anticipatory behavior

Anticipatory behavior is a suite of behaviors, expressed by animals during the appetitive phase, or before a desired outcome is acquired (10, 33). This class of behaviors is goal-directed, and aimed at acquiring desired outcomes (10, 33). In this paper, we will use the phrase anticipatory behavior to refer to animals' responses toward positive outcomes such as: breeding opportunities, positive social interactions, or food, and also behavioral opportunities to obtain primary reinforcers such as positive reinforcement training or enrichments (8, 9, 25, 41). Animals can express anticipation toward negative or unpleasant events as well (42, 43). Given the focus of modern accredited zoos is on providing positive quality of life and minimizing pain or distress (19, 20, 44), for the purposes of this paper we will focus on anticipation of positive outcomes.

As a welfare indicator, anticipatory behavior is thought to indicate an animal's own perception of its reward sensitivity (3, 45–47). Animals in a positive state of well-being are expected to exhibit frequent but low intensity anticipatory behavior toward known rewards. Animals in a more negative state may show infrequent but intense anticipation toward known positive outcomes (10). In essence, animals with fewer positively reinforcing opportunities will intensely anticipate the rare events they do receive. Intense anticipation may appear similar to an abnormal repetitive behavior such as pacing (10, 40). With further consideration of the timing, context, and location of the behavior, it may be possible to distinguish between abnormal behaviors and anticipatory patterns (40). Anticipatory behavior itself is neither a positive nor negative welfare indicator, rather the intensity with which it is expressed has been suggested as a graded welfare indicator for individual animals (9, 10).

Anticipatory behavior is not one single behavior, but rather a suite of behaviors an animal expresses ahead of acquiring a predictable reward to prepare to engage with the opportunity

(9), and can take several forms. The first is an increased level of activity ahead of gaining the desired outcome. A generalized increase in locomotion or activity has been documented across taxa ahead of predictable feedings (2, 25, 30, 31, 46–53). Alternatively, animals may sit and wait for the arrival of the anticipated outcome (23, 31, 54). Studies suggest differences between species in how anticipation is expressed (23, 31). Given anticipatory behavior is expressed across many species and is prone to developing under predictable conditions, it may be a complicating factor in interpreting behavioral data collected on animals living in human care. Behavioral data is often used to inform animal management decisions and draw conclusions about animal welfare (14, 55, 56). Understanding how anticipation influences animals' use of space, and varies with the timing and context of behavioral observations may thus have far reaching impacts on the care of captive animals.

Anticipatory behavior and space use

Anticipatory behavior develops from the learned association between a temporal or other cue and an outcome (4, 24, 26). As animals learn to associate a time or stimuli with an event, they are also likely to learn the location the event happens as well. When the timing and location of a positive outcome are both unpredictable, evidence suggests animals vary their space use and behavior (57). This response may be related to how animals have evolved to express appetitive/anticipatory behaviors—in measured amounts throughout the day. In many zoos, caregivers or keepers provide opportunities in predictable places due to necessary constraints on exhibit access. In the same way animals can learn to associate unintentional cues provided by keeper presence with positive events (26), animals learn to associate specific places with predictable events occurring there (22, 58, 59). The learned associated between a desired event and a location may result in the development of anticipatory behaviors. The relationship between anticipatory behavior and space use will depend on which style of anticipation individuals express. For example, animals showing a sit-and-wait anticipatory pattern may approach an area they are fed, then sit or stand nearby until they are fed (23, 31, 54). The space use by this individual would not vary measurably during the anticipatory period. Animals exhibiting more active anticipation may repeatedly approach areas an event happens while stopping to look, listen, or otherwise gather information about whether the desired event is about to occur (25, 60, 61). Information gathering behaviors are also likely to be directed toward where the event is expected to occur, specifically if the event is dependent on caretaker presence (8, 9, 61). If there are several vantage points from which animals can gather information (e.g., about the location of their keepers), animals may move rapidly between two points while anticipating, pausing to listen or watch at each (60). An animal exhibiting this type of anticipation may show space use in a limited area of their enclosure, perhaps along an apparently fixed path. The active form of anticipation is potentially more likely to be (mis-)identified as an abnormal repetitive behavior.

Studies of animal space use in zoos have utilized a variety of methods (62), and have been used to draw conclusions about animal welfare (63–66), enclosure suitability for a species (67, 68), and species level preferences or needs for substrates (68, 69). A common assumption of many space use assessments is that varied space use is preferable to animals using only a limited portion of an enclosure (62). As an anticipating animal may only be using a small portion of its exhibit, space use data collected in the anticipatory period may indicate a lower diversity in space use measures. This may be particularly problematic for studies assessing enclosure suitability or substrate preferences for a given species.

For example, anticipating dolphins have been observed spending time at the surface of their pools, waiting and watching for their trainers' approach (25). This study was designed specifically to measure anticipatory behavior. To this end, the researchers conducted observations immediately before training sessions when the dolphins received food as a reinforcer. In this example, the event the animals are anticipating is predictable to them, and the animals can gain additional information about the arrival of the event by spending time in a specific area (i.e., the surface of the pool). If researchers collected data in the same time frame but did not know the training was about to occur, the observed space use and behavioral patterns may have been interpreted differently. If the data were used to assess pool depth preference, the conclusions may have suggested dolphins prefer using the surface rather than deeper parts of the pools. The lower activity levels and use of a smaller area could also be interpreted as signs of poor welfare in the time period before the training session. It should be noted, the same animals were observed after the training sessions and showed different behavioral patterns and fewer surface-oriented behaviors than during the anticipatory period (25).

Identifying and accounting for anticipation in spatial data

Based on the previously described relationship between space and anticipation, several space use patterns may be of use in identifying anticipation. Clustered use of only a small area may indicate sit-and-wait form of anticipation occurring. Space use indicating a fixed path may be suggestive of the more active form of anticipation. Either form of anticipatory behavior would be expected to focus near where a desirable outcome is expected to occur. If the event is dependent on the presence of care staff, the animal's behavior may also be focused in areas where staff access the animal's enclosure.

Although we are discussing spatial data here, the distribution of data collection in time needs to be considered in determining whether anticipation might be influencing how animals use space. Balancing the start time of observations as much as possible throughout the day will help avoid undue influence of any specific management event. It is not uncommon for researchers in zoos to group data into broad pre-defined time periods (e.g., morning 10:00–12:00, afternoon 12:01–14:00, etc.,), depending on the animal's behavioral patterns or when it is most feasible to collect data. Data collection is then ideally balanced across all pre-defined time periods. This is a valid approach to addressing temporal variation in animal behavior. Within pre-defined time periods, however, the start times of observation sessions may not be balanced throughout the entire time period. For instance, perhaps due to timing constraints, 'morning' observations are started most days at 10:00, but the 'morning' time period extends through noon. If the animal receives its daily morning feed at 10:30 on most days, its behavior at 10:00 may differ from its behavior at 11:30. The behavioral observations throughout the entire day may be balanced between "morning" and "afternoon" time periods, while still overrepresenting an anticipatory period in the "morning". Thus, the animal's space use between 10:00 and 10:30 may not be representative of how the animal uses its enclosure when it is not waiting to be fed. Ensuring there is some variation in the start time of observations within broader pre-defined time periods will keep anticipation from unduly influencing the observed patterns of animal space use.

If space use is being used to determine whether anticipation is occurring, examining the animal's space use throughout the day at the same shorter timescale will be useful to verify space use patterns suggestive of anticipation. If a particular time period shows evidence of anticipation, it may be beneficial to exclude these data from analyses related to space use. Analyzing how animals use space outside of anticipatory time periods may provide a more independent measure of how the animal interacts with its enclosure or substrates independent of management events.

Anticipatory behavior and time

By definition, anticipatory behavior is dependent on time, as anticipation occurs before a predictable outcome (13, 24, 70, 71). Outcomes can become predictable to animals either by happening at approximately a similar time every day (53, 72, 73), being cued (intentionally or not, (8, 23, 26), or some combination of the two. Vertebrates have a well-developed internal clock, allowing them to develop a sense of when predictable events will occur in captive settings (24, 74). Reliable or semi-reliable cues animals learn in relation to caretaker behavior or environmental conditions can lead to anticipatory behavior as well (34, 75, 76). Feeding is commonly used to set, or entrain, circadian rhythms in laboratory studies (34, 77–79). The timing of feedings effectively set animals' internal clocks and circadian rhythms. Studies of rats and mice in laboratories have used wheel running as an index for activity level, and have quantified wheel revolutions throughout the day in relation to timing of feeds (80, 81). Measures of wheel running have provided insight into how anticipatory behaviors are expressed as predictable events approach. Specifically, anticipatory activity begins at low levels of intensity at time points before a predictable event, increases as the time of the expected event approaches, and then drops off suddenly when the desired event arrives. The sudden cessation of anticipatory behavior occurs when the animal is able to consummate the motivation the anticipation was directed toward (81). This structured temporal pattern of behavior can be contrasted with abnormal repetitive behaviors. Abnormal repetitive behaviors are typically described as functionless, and can result from varied etiologies (10, 82, 83). Based on the current understanding of these behaviors, there is no theory to suggest a temporal structure to when animals would express abnormal repetitive behaviors. Thus, this well-documented temporal pattern of behavior in anticipating animals shows the most promise as a diagnostically relevant factor for differentiating these classes of behaviors (40).

Food anticipatory activity has been documented in a wide variety of species (5, 8, 22, 23, 25, 27, 44, 48, 49, 56, 84, 85); however, the majority of this research was conducted in laboratory settings. Few studies outside of laboratories have examined how long before an event anticipation begins, nor what factors might impact the onset of anticipatory behaviors. Laboratory studies suggest food anticipatory activity tends to increase within an hour of expected feedings (13, 81, 82). Whether sit-and-wait anticipation is also expressed in a similar time frame is not known. Logistically, it may be more difficult to quantify changes to this style of anticipation over time. Increasingly rapid locomotion or location changes can be quantified, but measuring the intensity of an immobile behavior is challenging.

Animal behavior research has emphasized the value in understanding the relative importance of different resources to animals under human care (28, 84–86). As some resources will matter more than others depending on an animal's current state, animals can demonstrate behaviorally how much a given resource 'matters' to them by how much effort they will put in to obtain it (87, 88). Similarly, we may expect animals may express anticipation differently toward different resources. One study of domestic hens (*Gallus gallus*) demonstrated that the intensity of anticipation varies according to how much the reward is valued (89), and a study of a captive sea lion (*Zalophus californianus*) indicated the animal expressed more intense anticipation toward the first feed of the day compared to later feeds (9). As many non-domesticated species show seasonality of behavior and physiology associated with changes in behavioral drives, we may expect seasonal variations in anticipation as well. The extent to which an animal anticipates a particular event could vary seasonally, or the specific resources an animal anticipates may change throughout the year. For example, seasonal molting in birds increases the animal's energy requirements resulting in more food consumption (90, 91). Given the additional metabolic requirements of this process, animals may be more strongly motivated by food when they are undergoing a molt than at other times of the year. They may also exhibit more intense anticipation toward feedings than other opportunities during this time.

Identifying and accounting for anticipation in temporal data

Statistical methods already used to account for variation over the course of the day or study period, or to account for temporal autocorrelation may be useful for accounting for variation in behavior over time due to anticipation. Specifically, using generalized linear mixed models with a random effect for time of day to analyze behavioral data may help account for periods of significant anticipatory behavior in a data set, or account for variation in sampling through time (92). Assessing the response variables for temporal autocorrelation and including a variance structure accounting for this may also help account for temporal patterns within the data (92). As generalized linear mixed model methods can also tolerate uneven sampling across time periods, somewhat unbalanced timing of observations can be accounted for using this modeling method. Accounting for seasonal or annual variation is often done utilizing this method in other fields, and this may be useful for longer term zoo research as well (92, 93).

Ensuring observations are generally balanced throughout the day is another practical way to account for temporal variation in behavioral patterns. Even if timing of observations is grouped into pre-defined time periods, ensuring observation start times within each time period are varied can help balance out any anticipation captured in the observations. As previously stated, descriptions of how long before an event anticipation might be expected to begin are lacking outside of laboratory studies. As such, assessing behavioral data at a relatively short temporal scale, such as hour by hour, for signs of anticipation may be advisable. If a specific time period shows a much higher or lower activity level, determining whether any management events of particular importance to the animal occur around that time may help identify the behavior as anticipatory. When possible, determining whether the animal shows an increase followed by a sudden decrease in a particular behavior (e.g., walking or pacing) may be definitively used to identify a pattern as anticipatory. This approach would require repeated behavioral observations at a fine temporal scale, and may not always be feasible. Depending on the behavioral variables of interest for the study, excluding anticipatory periods from further analysis may be warranted.

As anticipation is directed toward a specific outcome, it is important to understand not only the temporal patterns of the behavior but also what management events happen and when they typically occur in a given day. As accredited zoos focus more on ensuring good welfare for animals in their care, most animals receive multiple daily positive opportunities in the form of feeding, enrichment, positive reinforcement training sessions, changing social groups, and other management decisions aimed at providing a varied and stimulating environment (20, 94, 95). Zoo animals may anticipate any of these events, but anticipation is most likely to develop for events that occur repeatedly, around approximately the same time, and/or are preceded by a cue or string of cues (9, 26, 76). Understanding the general time frame of daily management events an animal receives will therefore be a critical piece of information for understanding when the animal may be expressing anticipation.

Finally, if a concern is raised regarding a behavioral pattern that appears to be abnormal, the temporal patterns of the behavior may be useful in distinguishing between abnormal repetitive behaviors and intense anticipation. Specifically, if the behavior in question increases over a short period of time, and then decreases rapidly or stops after the arrival of a management event, there would be reason to conclude the behavior is anticipatory in nature. If it is not feasible to conduct detailed behavioral assessments in the time period the behavior is generally observed, an interview with care staff regarding the animal's regular daily schedule may help establish a timeline for when rewarding events occur for the animal.

Context

The factors we are referring to as 'contexts' in this review are any additional covariates that may impact study outcomes. Contexts or circumstances change in zoos throughout the day, weeks, or even months. As previously discussed, time and space are important and influence anticipatory behavior. For this paper, we are defining contexts as circumstances in a zoo that are out of the animal's control, and vary within space and time - essentially any covariate that can influence behavior. This is not a comprehensive list of all contexts animals experience in zoos, however we've attempted to broadly classify previous studies of relevant contexts here.

Anticipatory behavior and contexts

The impact of many specific contexts on anticipatory behavior have not yet been explicitly explored. In general, the direction of the relationship between a given context and anticipatory patterns will depend on the animal's level of reward sensitivity (10, 11, 33). The relationship between context and anticipation will also depend on whether the individual perceives the context as a positive or negative outcome (5, 23, 42, 96). We are including context as a separate factor from space and time, because although contexts vary within space and time, anticipation also varies between contexts (61, 97–99). In turn, variation in context will influence how anticipation is expressed in time and space. For instance, an animal with varied enrichment may demonstrate its lower reward sensitivity through less intense anticipation (45, 100).

Human contexts

One context that receives significant attention in all zoological institutions is the effect of humans. Visitors and care staff are present on a daily basis. Repeated interactions with humans may be considered human-animal relationships (HAR), and the relationships between animal care staff and the animals they care for can have implications for animal behavior and well-being (101–105). Studies have shown that HARs can have a positive, negative, or neutral effect for animals and depend on the quality and quantity of interactions between two individuals (105). A case study of two zoo animals suggests that animals under human care can find social interactions with non-caretaking humans positive, even when the interaction resulted in no primary reinforcement (23). This study demonstrated this social interaction was rewarding enough to lead to the development of anticipatory behaviors when the interaction followed a reliable signal (23). Besides the quality of an animal's relationship with its caretakers, keeper presence is one of the major factors that influences daily conditions animals experience (106). Keeper presence is often associated with positive events for the animal, and animals are generally highly attuned to cues related to their keepers (26, 105, 107). The arrival or presence of caretakers likely shapes daily patterns of animal behavior. The majority of an animal's feedings, enrichments, or training sessions will occur within a short time of a keepers' arrival (49, 108). For instance, dolphins anticipating positive reinforcement training sessions orient themselves according to keeper presence and activity (25). The context of care staff presence may therefore influence the timing and spatial components of animals' behaviors. Thus, an individual animal's experience of its relationship with its caretakers and the frequency of keeper visits both have potential to impact anticipatory patterns.

Zoo visitor presence is known to impact animal behavior in various ways. Interactions between visitors and zoo animals are a subset of human-animal relationships studied in zoos known as the visitor effect. The effect of visitor presence on animals is well–documented (109, 110). The nature of the impact that visitors have on animal behavior varies. Studies have shown varying levels of negative impact associated with high visitor density, including increased corticoid concentrations (111, 112), increased hiding behavior (113), increased abnormal repetitive behaviors (114, 115), and increased intra-group aggression (116). The impact of crowd size is variable, however, with some studies finding a negative relationship and others finding no impact, even in the same species (109, 117). Animals' response to visitor presence is likely influenced by species and individual personality (117, 118). To date, little or no research we could find has been done investigating the relationship between visitor numbers and anticipation in zoo settings. This may be an avenue for future investigation. How an animal's anticipatory patterns change with visitor numbers is likely to depend on whether it perceives visitor presence as aversive or enriching. The predicted relationship between reward sensitivity and intensity of anticipation can be useful in predicting how animals' anticipation may vary with visitor numbers (10). Animals finding visitor presence stressful would be expected to exhibit more intense anticipation under high visitor numbers. Animals experiencing visitor presence as enriching may exhibit minimal anticipation when visitor numbers are high. The potential for correlation between higher visitor numbers and events the animals perceive as high value may complicate such a study. Specifically, if trainings or feedings are advertised to zoo visitors, the timing of increased visitor numbers at the animal's exhibit and the time leading up to the management event may be confounded.

Social contexts

The social context of animals also impacts many aspects of how they interact with their environments (see (119) for an in-depth review). The social context of an animal includes intra-specific interactions with conspecifics. The nature of intra-specific interactions is expected to vary with the size and composition of the group (120), as well as the individual temperaments of the group members (120, 121). An animal's social context may also include any individuals of another species with which the animal shares space (122, 123). Social context does not only include animals with physical access to each other, as both conspecifics or heterospecifics within the perceptible range of an individual animal may impact its behavior. For example, okapi (*Okapia johnstoni*) with visual access to conspecifics exhibit more pacing (124), and the sex-ratio of animals in surrounding pens impacts breeding behavior in giant pandas (125). In a mixed-species example, alarm calling and vigilance in brown capuchins (*Cebus apella*) decreased with the addition of a visual barrier between the primates and a small felid in a nearby exhibit (126). Studies of anticipation and social contexts in zoo animals are limited; however, laboratory studies indicate social interactions can have significant impacts on anticipatory patterns of individuals (46).

Environmental contexts

It has long been recognized that inappropriate environmental conditions can compromise animal well-being. Due to this, zoo scientists are increasingly interested in empirically assessing the environmental conditions animals experience to ensure animals can achieve positive well-being. Assessments have examined animals' responses to the myriad environmental conditions they are subject to, such as artificial lighting (127, 128), sound levels (129–131), or the thermal environment (132, 133). Such environmental measurements may be the main focus of the study, or included as a covariate expected to impact animal responses (134, 135). Interest in the impact of complex changes to normal environmental conditions is also increasing, with more research being conducted on events held at or impacting zoos (136–139).

The most common method to provide changes to the environment is environmental enrichment. Environmental enrichment is a component of animal husbandry that aims to provide a dynamic environment through varied behavioral opportunities for animals under human care (62, 140–144). Environmental enrichment can take many forms, including feeding strategies, sensory, social, structural, and cognitive enrichments (143, 145). Giving enrichment daily is common, but the type of enrichment, frequency, and timing can vary between enclosures, species, and zoos. Type, frequency (times throughout the day), timing, and location of enrichment can be an essential context to consider when collecting behavioral data. Studies in farm animals indicate a variety of animals exhibit anticipatory behavior ahead of receiving environmental enrichment opportunities (5, 30, 144). Enriched environments are generally associated with indicators of positive well-being in animals, such as increased engagement with their environments (146–148), positive judgment biases (149, 150), and play behaviors (41, 151, 152). Enrichments providing problem solving opportunities have also been associated with lowered intensity of anticipatory behaviors (60), as well as other indicators of positive well-being in animals (153, 154).

Identifying and accounting for anticipatory behavior in relation to contexts

The contexts an animal experiences are likely to interact with anticipation by modulating the animal's overall reward sensitivity (10). As already stated, any outcomes the animal finds to be positive are candidates for the animal to express anticipation toward, and the more of these an individual experiences the less intense overall anticipation is expected to be. Thus, when a study aims to alter one or more contexts an animal experiences, gaining as complete a picture of what the individual's 'normal' day comprises ahead of any changes is critical. This is already a common feature of many studies in zoos, with baseline data collected ahead of any manipulations to the environment or animal management. Alongside the collection of baseline behavioral data, understanding the timing, frequency, and individual preferences for various contexts and events study animals experience in their daily lives can provide a more complete understanding any resulting changes observed during the study.

Context is also included here as it is expected to vary in both space and time, suggesting animals may be experiencing their environments differently throughout the course of the day. This seemingly basic statement has important implications for anticipatory patterns of individual animals. It is common for zoo animals to be shifted into publicly visible spaces when the zoo opens, and they receive a portion of their daily diet and novel enrichment for the day. By later in the day, the enrichment has been engaged with or emptied of food, and the animal's diet may be consumed. The environment the same animal experiences 4 h after shifting may be significantly different in terms of context than the environment it shifted into in the morning, with potentially fewer behavioral opportunities available (26, 39). Thus, the biological relevance of the animal's environment is likely to change throughout the day. The timing of events in relation to one another and potentially the order of events may all be important contexts to consider as well.

Anticipation may be an unrecognized source of variations among behaviors of group-living individuals, as each individual has the potential to experience a given context differently. For example, a more dominant group member may have the opportunity to exploit feedings or enrichments first, or subordinate individuals may not receive as many positive social interactions with other group members. Less dominant animals may thus be expected to display more intense anticipation on average than more dominant individuals. Ruling out whether this is the case may help account for results when a change is observed in behavior at the group level; but, the outcome is driven by a single animal's response. Considerations of context will necessarily vary according to what the overall question of a study is.

Conclusion

Throughout this review, we discussed space, time, and context separately—but in practice, all of these factors are interconnected. How animals use space or experience different contexts are constantly changing through time. Understanding space use, temporal patterns, or contexts influencing animal behaviors requires concurrent understanding of each of the other factors in many cases.

We have identified several specific patterns of how anticipatory behaviors are expressed in relation to space, time and contexts, based on reviewing the existing research. Specifically, if a behavior is anticipatory, it would be expected to (1) occur in an area proximal to where a positive event occurs (2) increase in frequency or intensity as the time of a predictable positive outcome approaches (3) cease to be expressed when consummation of the motivation occurs and (4) be modulated by other contexts expected to change individual's reward sensitivity (e.g., decrease in intensity with increased opportunities to obtain rewards and vice versa). These patterns can be useful for identifying anticipation in animals living under human care.

The extensive body of research into how animals use their spaces, respond to changes over time, and other contexts influencing animal behavior have been a major part of the zoo animal welfare field. As the focus of animal management and care moves toward the goal of providing more choice, control, and complexity for animals, the methods used for measuring how animals respond to these changes need to shift as well. By integrating spatial and temporal considerations explicitly into how we measure animal behavior, we can improve our understanding of the prevalence of anticipatory behaviors, and clarify how these behaviors may have inadvertently shaped our conclusions about animals' preferences and requirements.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

BK conceptualized the manuscript. BK, KC, CE, CT, and EP wrote the manuscript. BK, KC, CE, CT, EP, and JW provided significant edits to the manuscript. JW is the principle investigator and provided logistical support to all contributors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dairy cows did not rely on social learning mechanisms when solving a spatial detour task

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As herd-living animals, cattle have opportunities to observe and learn from others. While there is evidence of simpler processes of information transfer in cattle (social facilitation and stimulus enhancement), true social learning mechanisms in cattle remain largely unexplored. This study aimed to investigate if dairy cows possess cognitive abilities to acquire new behavior through social learning in a spatial detour task. Thirty-two dairy cows (ages 2–9 years) participated in the study. A food reward was placed behind a U-shaped formation (4 x 2 m), allowing the cows to see but not reach the reward without first detouring around the obstacle. The U-shape provided two routes (∼18 m walking distance) to the reward, of which one was used for demonstration. Two cows were demonstrators and 30 cows were divided into two groups, assigned as either observers of demonstration ($n = 15$) or controls not observing demonstration ($n = 15$). Cows had three attempts (trials) to solve the task. Response variables were: success, latency to reach the reward, concordance in choice of route to detour, and time spent facing the test arena before each trial started. The study found no significant differences in success or latency between observers and controls, although observers spent a greater proportion of the time before trials facing the test arena. However, successful observers tended to be faster than successful controls. Individual cows were generally consistent in their choice of route, and cows choosing the demonstrated route were significantly faster than cows that did not. Success in solving the task decreased over trials, likely due to decreasing food motivation. Age had a significant effect on success in 2nd and 3rd trial, with younger cows being more successful. The lacking effect of treatment on success suggests that the age effect may be explained by a higher motivation, rather than social learning. Adding to the sparse knowledge of social learning in farm animals, these results indicate that cows did not utilize social learning mechanisms when solving the detour task. Future research should focus on clarifying whether cattle possess cognitive abilities necessary for social learning, as well as if /when social learning is a primary strategy.

KEYWORDS

cognitive task, cattle, animal welfare, social transmission, animal learning, cognition, bovine, observational learning

Introduction

Animals can acquire new behavior through individual and social learning. Individual learning can occur through an individual's own experience, e.g., of trial and error, whereas social learning is influenced by observing or interacting with others (1, 2). In an unpredictable environment where the consequences of failing through trial and error may be dire, learning by observing others can be a beneficial strategy to acquire new information at reduced costs (1). Observing others may also facilitate individual learning in situations where an animal's behavior is influenced by the observation but the actual learning is a direct result of the animal's own experience, rather than the observation itself. For example, synchronized behaviors rely on social facilitation where the motivation to perform an already established behavior is increased after observing other individuals performing that same behavior (3). Social facilitation can thus be considered a social influence on behavior, not a form of learning, as it only leads to an increase (or decrease) in the performance of an existing behavior (3, 4). Through stimulus enhancement, observing other individuals interact with a specific stimulus (e.g., a novel drinker) can increase the motivation of an animal to investigate that same stimulus, subsequently creating an opportunity for individual associative learning of how to operate it (4, 5). These processes of information transfer that facilitate individual learning are collectively referred to as social transmission and differ from true social learning (6).

Evidence of social learning in cattle is scarce. Previous studies have focused on the transmission of information from parent to offspring, or from older to younger individuals. For example, calves develop preferences for pasture locations and habitats based on early-life experiences of grazing together with their dam or foster dam (7), and naïve heifers are quicker to start grazing when grouped with older, pasture-experienced cows compared to when grouped with naïve peers (8). These findings can, however, be explained by simpler processes of information transfer such as stimulus enhancement and social facilitation (i.e., social transmission mechanisms), and are thus not evidence of true social learning. In studies on sheep, another grazing livestock, lambs have been shown to learn which food to eat and which food to avoid from grazing with their dams [e.g., (9, 10)]. Such observations are indicative of social learning mechanisms if the lamb (or calf) learns to eat or avoid a novel food resource, which it has no prior experience with, and expresses this behavior without the parent present. Social influences (regardless of the cognitive mechanisms involved) on feed intake, feed selection as well as the sampling of novel, potentially toxic food decrease the risks associated with trial-and-error learning in foraging (11).

Other studies have investigated the transmission of information between peers. Heifers presented with an operant task of pushing a panel to access a food reward do not improve in learning the task after observing it performed but spend longer time engaging in the task if they first observe a demonstration (12), hence a clear example of stimulus enhancement. Naïve heifers will be more successful in finding feed locations in a maze when accompanied by a trained peer (13). Cows can be socially influenced by the response of other cows when determining what distance to keep from an aversive handler (14). Likewise, responses to virtual fences can also be socially facilitated, with cattle staying within the intended zones based on the response of peers to auditory and electrical cues (15). Recently, Stenfelt et al. (16) found that a calm companion lowered fear in small groups of dairy cows ($n = 4$) when exposed to the novel and aversive stimulus of the opening and closing of an umbrella. Like the findings of transmission of information between cattle parent and offspring, these findings of transmission between cattle peers can be explained by the simpler processes of stimulus enhancement and social facilitation (i.e., social transmission mechanisms). As mentioned, a social influence on the performance of an existing behavior can be distinguished from the learning of a new behavior (3, 4). Hence, more research is needed to establish whether cattle have, and make use of, the ability for true social learning when acquiring new behavior from conspecifics, whether parents or peers.

Social learning requires cognitive abilities of higher complexity than social transmission (6, 17, 18). Thus, the copying of an individual's motor pattern, also referred to as imitation, requires the observing animal to match the visual representation of the demonstrator with its own proprioceptive control (6, 18). Reproducing the results of an individual's behavior rather than the precise behavior itself, also referred to as goal emulation, requires the observer to make a connection between the insights gained from observing and the observer's own motivations (6, 19). Solving a spatial detour task is a method previously used to investigate social learning in animals [e.g., (20–25)]. In a detour task, the animal must navigate around an obstacle to reach a certain goal, e.g., a reward. This requires momentarily moving away from the goal, i.e., increasing the distance to the reward, in order to reach it. For gregarious ungulates, like cattle, who in their natural environment would navigate over large distances and through changing terrains, a spatial task seems to be of greater biological relevance than, e.g., an operant task which is not a part of their natural environment. Hence, exploring social learning in this spatial context would give valuable information about the cognition of cattle.

The information transfer between conspecifics (e.g., cow to cow), as well as heterospecifics (e.g., human to cow), can be studied by allowing an animal to observe a trained demonstrator performing the spatial detour task. This has not yet been used in social learning experiments with cattle, but with several other species and with varying results. For example, domestic dogs (*Canis lupus familiaris*) have been shown to use inter-species social learning when solving a detour task after observing a human demonstrator, although they did not copy the demonstrator's exact route (20). Sanctuary-raised dingoes (*Canis lupus dingo*) were tried in an equal experiment and proved more successful than domestic dogs in solving the detour task, although their performance was unaffected by a human demonstrator (21). The lack of inter-species social learning in dingoes indicates that the ability of dogs to learn from human demonstrators in a detour task may be a result of the increased attentiveness to, and ability to read, human communicative signals following the domestication process (26). Being a domesticated species, it is likely that cattle too, at least to some extent, have been selected to pay attention to human body language.

Like domestic dogs, domestic goats (*Capra aegagrus hircus*) appear to use inter-species social learning, as they were significantly helped in solving the detour task by observing a human demonstrator (23). This is in contrast with the results of studies on domestic horses (*Equus caballus*), which have not been shown to benefit from demonstrations from humans or conspecifics (22, 24). The presence of a conspecific behind the obstacle has, however, shown to impact the detour strategy of horses (25), potentially indicating that social companionship (or lack thereof) may be an important aspect to consider when designing a detour task. As the cognitive mechanisms of domesticated ungulates appear to vary between species, it is possible that cattle may possess the ability to learn from observing others. Learning more about the social cognition of cattle will help us in our understanding of their social environment and provide insight into how cattle acquire new knowledge and behavior. This study aimed to investigate if dairy cows possess the cognitive abilities to acquire new behavior through intra-species social learning in a spatial detour task. The main hypothesis was that cows observing a trained demonstrator cow performing the detour task would be more successful in solving the task and do so with shorter latencies compared to control cows that did not observe such demonstration.

Materials and methods

Ethical considerations

The details of the experiment were assessed and approved by the "Board for Animals in Research and Teaching" at SLU, Sweden. Of the three experimenters who took part in the training and testing, two had an education in responsible use and treatment of animals used in research and supervised the third experimenter (MSc student). All methods used and care for the animals complied with national legislation on animal experimentation by the Swedish Board of Agriculture (27) and met the ARRIVE guidelines (28) as well as complied with the ethical guidelines proposed by the Ethical Committee of the ISAE (International Society of Applied Ethology) (29). The director of Uddetorp Agricultural School and the staff involved were informed about, and agreed to, the details of the study.

Animals and housing

Thirty-two dairy cows of Uddetorp Agricultural School in Skara, Sweden, participated in this study. The training of cows and data collection took place over the course of 3 weeks in June of 2021. During this time, the cows were loose housed in a free-stall cowshed, with at least 12 h of pasture access per day. In addition to grass from being pastured, cows were fed a partial mixed ration with concentrates in transponder-controlled feeders and had *ad libitum* access to water. The cows were a mixture of Swedish Holstein ($n = 20$) and Swedish Red ($n = 12$), with the uneven distribution between breeds due to availability on the farm. The cows were of varying age (2–9 years), in varying parity (1^{st}-6^{th} parity), and in various stages of the lactation cycle. Cows that had recently calved were given at least 1 week to recuperate before joining the experiment, and cows expected to calve within a week from the day of testing were excluded.

Demonstrators, observers and control cows

The dominance relationship between demonstrator and observer has been suggested to be important for the facilitation of social learning (30), with dominant animals making better demonstrators (1, 22). In previous studies on horses, demonstrators have been selected based on the results of investigations into the dominance hierarchy (22, 25). Such an investigation was not feasible within this study, instead, the demonstrators were chosen based on brief behavioral observations during interactions with herd members. On three separate occasions, the experimenters visited the herd on pasture as well as in the cowshed and assessed (i) the success of initiating movement of one or more followers, (ii) the willingness of the cows to approach and interact with the handlers, and (iii) winning agonistic encounters between herd members. When conducting the first two observations, the experimenters walked around in the pasture and noted which cows voluntarily approached the experimenters. The experimenters further noted which of the approaching cows seemed to attract a following of other cows previously reluctant to approach on their own. Lastly, the experimenters noted agonistic interactions that occurred during this time, e.g., butting (31, 32). When conducting the third observation, the experimenters visited the cows in their free-stall cowshed and again noted which cows would approach, which appeared to initiate the approach of other cows, as well as agonistic interactions. The visits lasted for ∼15–20 min. Two cows met the stated requirements and were selected to participate as demonstrators. Both demonstrators were 3 years old, one Swedish Holstein and one Swedish Red.

The remaining 30 cows were divided into two groups, balanced primarily on breed and age, but also with some consideration to brief observations of behavior displayed during the habituation process (see Habituation to experimental venue for details) e.g., agonistic encounters between group members eating from or approaching the same bucket, if a cow seemed shy/fearful of the experimenter refilling and moving buckets around within the experimental venue, and if a cow seemed highly motivated to obtain the food reward or was observed grazing before approaching the refilled buckets. The two groups were then randomly assigned as either observer ($n = 15$) or control ($n = 15$).

Experimental design

The cows were presented with a yellow bucket that contained a food reward (pelleted concentrates). This specific concentrate was part of the cows' partial mixed ration and was chosen as the food reward per the suggestion of the farm staff, as they knew the cows to be highly motivated to obtain it. The type of bucket (Red Gorilla flexible TubTrug 26 L) and its yellow color were chosen to ensure the reward bucket differed from the black buckets with metal handles typically used at the farm, and to ensure that the cows could differentiate it from the green grass (33). The yellow reward bucket was positioned behind a U-shaped obstacle of metal cattle gates, which allowed the cow to see the bucket but not reach it without first going around the gates (Figure 1), i.e., solving a spatial detour task. The aim was to assess differences in the response (latency) and success rate of completion of the task over three consecutive trials (i.e., three attempts carried out on the same day), between cows in a treatment group that first observed a trained demonstrator cow solve the task and reach the reward bucket, and cows in a control group that did not receive the demonstration.

Habituation to experimental venue

The experimental venue consisted of a fenced-off section of the cows' regular pasture. All cows were habituated to the experimental venue and the yellow reward buckets in small, randomly assembled groups of 3-4 cows. The groups were driven into the test arena where four yellow buckets, containing a handful of food each, were randomly distributed across the experimental venue. As the cows finished the content of a bucket, it was instantaneously refilled and moved to a new location within the test arena. Thus, the cows were continuously seeking food from the presented reward buckets. All cows were habituated in this way for 10 min on two separate occasions. The cows were considered habituated if, when released into the test arena alone, they immediately walked up to and ate from the reward bucket in its designated place (without the cattle gates present) during minimum 30 s. Before the detour test took place, all cows were pre-tested once on the habituation criterion individually.

Training of demonstrators

The two demonstrator cows were trained to follow one of the experimenters carrying a black bucket containing food (pelleted concentrates), along the demonstration route (Figure 1). The demonstrator was regularly allowed to eat from the black bucket to reinforce her motivation to follow the experimenter. When reaching the yellow reward bucket behind the cattle gates, a handful of food was dropped into the reward bucket and the demonstrator was allowed to eat from it before continuing with a second and third identical lap, before being placed in the demonstrator pen (Figure 1). The demonstrators were trained for ∼20 min each on two separate occasions prior to the detour test. The demonstrators were considered trained and habituated if they, during at least three consecutive laps around the test arena, consistently followed the experimenter and could shift their focus to eat from the yellow reward bucket and then back to following the experimenter again.

Detour test

The detour test took place on three separate days. The 30 cows were divided into three subsets which were balanced on treatment, and each subset was tested on one of the three test days (day 1: $n = 10$, day 2: $n = 10$, day 3: $n = 10$). All cows were tested within a week of complying with the habituation criterion. The cattle gates were assembled out of sight for all cows, and the demonstrator was placed in its demonstrator pen (either to the left or the right, which was balanced to control for laterality of the cows) with a large tub of water and an empty white food bowl. The test subject (i.e., observer/control) was collected from the cowshed and driven to the test arena by use of negative reinforcement if needed (e.g., gently tapping on the cow, using soft sounds, and/or gesticulating to encourage walking). Extra care was taken to avoid stressing the cows and to make sure they arrived at the test arena as calmly as possible. Observers and controls were alternated throughout the test and were always given either left-sided or right-sided demonstrations, never both. The side of the demonstration (i.e., left/right) was balanced over test days.

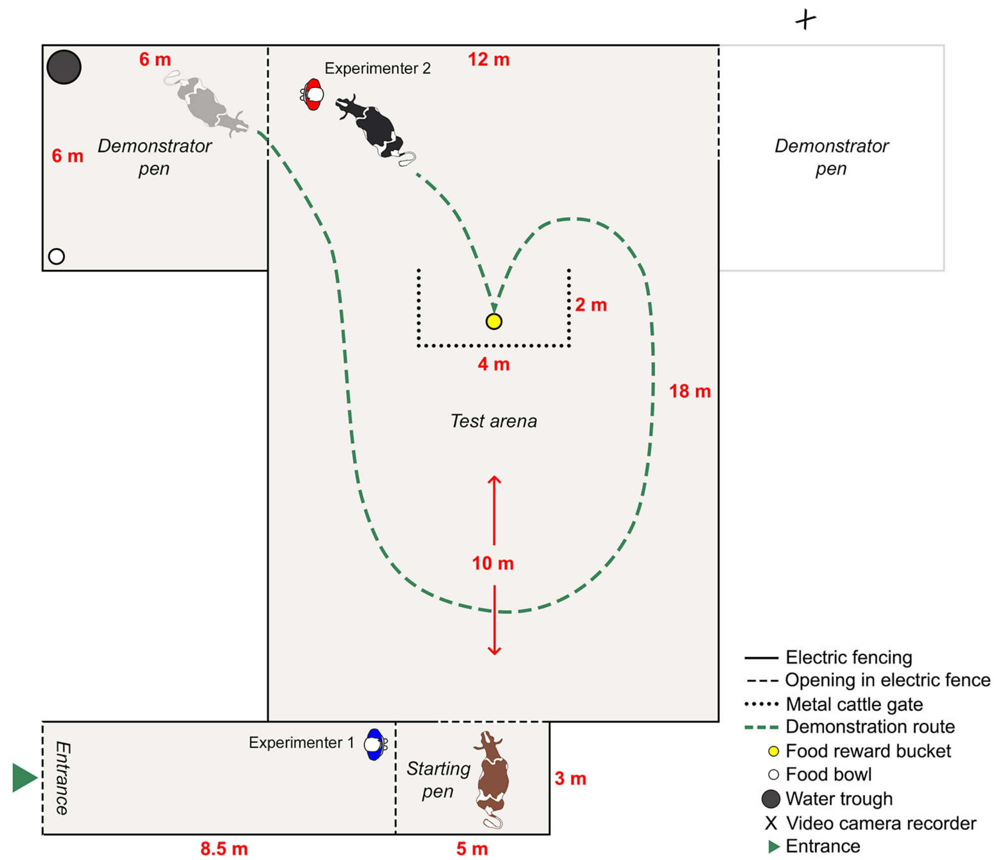

FIGURE 1
The layout of the experimental venue during a right-sided demonstration of the spatial detour task, where the black cow is the demonstrator and the brown cow the observer. The grayed-out demonstrator pen was used for left-sided demonstrations, during which the video camera was moved to the opposite side of the test arena.

Test procedure for observers

The observer cow was placed in the starting pen by experimenter 1, who then remained on the left side of the starting pen (Figure 1). The demonstrator cow was led out onto the test arena by experimenter 2 and the demonstration began. As per prior training, the demonstrator followed experimenter 2 and the black bucket along the demonstration route and stopped to eat a handful of food from the yellow reward bucket before continuing with a second and third lap (i.e., all observer cows received three demonstrations before their first trial). After the third lap, the demonstrator was led back to the demonstrator pen, where experimenter 2 dropped a handful of food in the white food bowl to keep the demonstrator cow occupied while securing the arena with electric fence gate handles. Experimenter 2 then walked over to the yellow reward bucket within sight of the observer, dropped a handful of food in the bucket with a rattling sound to ensure the attention of the observer, and walked over to release the observer from the starting pen. Experimenter 2 walked the demonstration route (as to not provide any human demonstration of the opposite route), i.e., behind the cattle gates on the demonstration side and exiting on the side of the demonstrator pen (Figure 1).

To release the observer from the starting pen, experimenter 2 opened the gate and stepped into the starting pen on the cow's right side (opposite of experimenter 1) for a symmetrical pressure of both sides so as to not influence the choice of route. Both experimenters remained at the starting pen during the trial. The latency to reach the reward bucket was measured from the moment both hind legs of the observer were positioned in the test arena until her muzzle reached the bucket. If the observer did not take the demonstration route, or if she failed to reach the bucket within 90 s, the attempt was considered unsuccessful, and the observer was retrieved to the starting pen using the reward bucket as a motivator. When the observer was back in the starting pen, the reward bucket was returned to its designated place and a one-lap demonstration was performed before the observer was released for a new trial. If the observer took the demonstration route and reached the reward bucket within 90 s, the attempt was considered successful. The observer was then retrieved to the starting pen using the reward bucket and released for a new trial as soon as the reward bucket had been returned and refilled. Observer cows were given a total of three consecutive trials (attempts) within the test day.

Test procedure for controls

The control cows were placed in the starting pen where they waited 3 min, corresponding to the duration of a three-lap demonstration for observers. During this time, the demonstrator remained in its pen, and experimenter 2 waited outside the demonstrator pen, next to the gate (Figure 1). Before the control was released, experimenter 2 walked to the yellow reward bucket within sight of the control, dropped a handful of food in the bucket with a rattling sound (as to allow for the same stimulus enhancement as for observers), and walked over to release the control from the starting pen. Experimenter 2 walked the same route as a demonstrator cow would have; behind the cattle gates on the demonstration side and exiting on the side of the demonstrator pen. The control was released in the same way as the observer, with one experimenter on each side of the starting pen. After 90 s or upon reaching the reward bucket, regardless of the route taken, the control was retrieved to the starting pen using the reward bucket, where she waited 1 min (corresponding to the time of a one-lap demonstration as to offer controls the same amount of time to observe the test arena and the spatial problem, as the maximum wait between observer trials) before being released into the test arena for the next trial. Control cows were given a total of three consecutive trials (attempts) within the test day.

Recording

Demonstrations and trials of both treatment groups were continuously recorded using a video camera on a stationary tripod. The completion of the detour task (yes/no), the chosen route (demonstration/opposite), and the latency from release to completion (s) were recorded on-site and later confirmed by use of the video footage. The video footage was also later used for continuous recording of the time individual cows of both treatment groups spent facing the test arena in the time before each trial started. The time spent facing the test arena was recorded as the total duration of the cow having her head lifted from the ground and facing the test arena/spatial problem at a maximum of $\sim 22.5^\circ$ away from the center of the test arena. The 22.5° on each side of the center together made up an area of $\sim 45^\circ$ in total. In practice, this covered the width of the test arena and ~1–1.5 m of both demonstrator pens to where the video camera was located (Figure 1). Concordance in route (demonstration/opposite) was recorded as the number of trials in which the cow repeated the route taken in her first successful attempt, as an indication of a potential learning process. Concordance could vary between 0 (if route in both second and third trial differed from first trial) and 2 (if route in both second and third trial were the same as in first trial).

Data editing

The sample size for the statistical analysis of success was 30 cows ($n = 15$ observers, 15 controls) and a total of 90 trials ($n = 45$ observers, 45 controls). Latency was recorded for all trials in which the test cow solved the task within 90 s. The sample size for the statistical analysis of latency was thus 29 cows ($n = 14$ observers, 15 controls) and a total of 61 trials ($n = 32$ observers, 29 controls). Some video footage was unfortunately lost due to technical difficulties, thus the sample size for the statistical analysis of time spent facing the test arena was 27 cows ($n = 13$ observers, 14 controls) and 79 trials ($n = 38$ observers, 41 controls). Concordance in route over trials included test cows that reached the bucket during their first attempt and thus had a learning opportunity in first trial with two chances to repeat the success in the following trials. The sample was further standardized by only including cows that passed the obstacle in both second and third trial, and thus had the opportunity to approach the reward bucket if they wanted to. The final sample size for the statistical analysis of concordence in route was thus 20 cows ($n = 10$ observers, 10 controls) and a total of 60 trials ($n = 30$ observers, 30 controls).

Statistical analysis

All statistical analyses were performed in R version 4.1.0 (34) with RStudio version 1.4.1717 (35), using the packages lme4 (36), emmeans (37), nnet (38), MASS (38), car (39), DHARMa (40), Rmisc (41) and tidyverse (42). *P*-values below 0.05 were considered significant.

Success

The success of each cow in each trial was recorded as a categorical variable with three levels: complete success (reaching bucket within 90 s through demonstration route), partial success (reaching bucket within 90 s through opposite route), and no success (failing to reach bucket within 90 s regardless of route). Two generalized linear mixed-effect models (GLMMs) were employed to investigate effects of treatment (categorical variable with two levels: observer/control), trial number (categorical variable with three levels: first/second/third), age (numeric variable, mean $\pm$ SD $= 3.7 \pm 1.5$ years) and breed (categorical variable with two levels: Swedish Holstein/Swedish Red), with cow as a random factor. Each model included a binomial response of success (i.e., success vs. no success): the first model considered both partial and complete success (vs. no success), whereas the second model only considered complete success (vs. partial and no success). This yielded two analyses, one on the success of reaching the reward bucket (i.e., solving the detour task regardless of route), and one on the success of doing so through the demonstration route (i.e., solving the detour task using the demonstrated route). This model type was chosen over multinomial models to account for repeated measures on each cow, as each cow was tested in three trials. As the primary response variable was binary (i.e., successful or not), data was analyzed using a logistic regression. Estimated marginal means (EMMs) were calculated for all fixed effects. As initial analyses of success revealed an unexpected decrease in success over trials, a *post hoc* analysis was carried out analyzing first trial separately. For this, a multinomial model was used, including fixed effects of treatment, age and breed. EMMs were calculated and used for pairwise comparisons of all fixed effects.

Latency

Latency was defined as the time it took for the cow to reach the yellow reward bucket from the moment both hind legs stepped onto the test arena. To account for the repeated measures on each cow, a linear mixed-effects model (LMM) was employed to investigate fixed effects of treatment, trial number, age (mean $\pm$ SD $=$ 3.4 $\pm$ 1.6 years), breed and choice of route (categorical variable with two levels: demonstration/opposite), with cow as a random factor. EMMs were calculated for all fixed effects.

Facing of test arena

The time spent facing the test arena was considered as a percentage of time before each trial (as this time varied depending on if one-lap, three-lap, or no demonstration was performed) and was analyzed using a LMM considering fixed effects of treatment, trial number, age (mean $\pm$ SD $=$ 3.7 $\pm$ 1.6 years) and breed, with cow as a random factor. EMMs were calculated and used for pairwise comparisons of all fixed effects.

Concordance in route

Concordance in route was defined as the number of times the cow repeated the route taken in her first successful attempt, and summarized as an individual score. The total score (0-2) for each cow was analyzed in an ordinal logistic regression model considering fixed effects of treatment, age (mean $\pm$ SD $=$ 3.5 $\pm$ 1.8 years) and breed. EMMs were calculated and used for pairwise comparisons of all fixed effects.

Results

Success

There were no significant differences between treatment groups in the success of solving the detour task (Table 1) or in the choice of route (Table 2). In first trial, 27 out of 30 cows (n = 14 observers, 13 controls) reached the yellow reward bucket within 90 s regardless of route (Figure 2) and solved the detour task (i.e., achieving complete or partial success). Of these 27 successful cows, 15 individuals (n = 6 observers, 9 controls) took the demonstration route (i.e., achieving complete success) in first trial. Although the overall success of solving the task (i.e., regardless of route) was high in first trial, it significantly decreased with the following trials (Figure 2). The fitting of the models showed a significant effect of age on success, with younger cows performing better than older cows in the overall success of solving the task (Table 1) but not in doing so through the demonstrated route (Table 2). This effect of age was insignificant in the *post hoc* analysis of first trial. The *post hoc* analysis further revealed an insignificant effect of treatment (estimate $\pm$ SE $=$ 0.35 $\pm$ 0.17, t = 2.01, p = 0.07), suggesting that the controls were more inclined to take the demonstration route, while the observers were more inclined to take the opposite route in first trial (Figure 3).

Latency

In 61 out of 90 trials, 29 test cows (n = 14 observers, 15 controls) were overall successful in solving the task (i.e., regardless of route) and thus had a latency to reach the reward bucket recorded. In these successful trials, observers had a tendency for shorter latencies than controls (Table 3). Furthermore, there was a significant effect of choice of route on latency, where cows of both treatment groups that took the demonstrated route were significantly faster to reach the reward than cows using the opposite route (Figure 4). Trial had no effect on latency, indicating that cows did not become increasingly faster (or slower) in solving the task over trials (Table 3).

Facing of test arena

All test subjects (n = 13 observers, 14 controls) spent time facing the test arena before each trial started. Observers spent a greater percentage of the total time before trial (i.e., during demonstration) facing the test arena (mean $\pm$ SD $=$ 45.66 $\pm$ 20.10 %) than controls did (mean $\pm$ SD $=$ 35.56 $\pm$ 17.85 %). The difference between treatment groups was marginally significant (estimate $\pm$ SE $=$ 10.4 $\pm$ 5.2, t = 2.0, p = 0.06).

Concordance

Concordance in route over trials indicated that individual cows of both treatment groups (observers: n = 10, mean $\pm$ SD = 1.30 $\pm$ 0.82, controls: n = 10, mean $\pm$ SD = 1.50 $\pm$ 0.71) were generally consistent in their choice of route to detour. There was

TABLE 1 Summary of the mixed-effects logistic regression on success of solving detour task regardless of route.

Variable	Levels	*n*	EMM	$SE_{(EMM)}$	Asymp. 95% $CI_{(EMM)}$	Df	*p*	
Age	Continuous	30	1.09	0.38	0.34–1.84	1	0.02	*
Breed	Holstein	19	1.23	0.46	0.33–2.13	1	0.66	ns
	Red	11	0.95	0.54	−0.10–2.00			
Treatment	Observer	15	1.32	0.52	0.30–2.34	1	0.45	ns
	Control	15	0.86	0.46	−0.05–1.76			
Trial	First	30	2.66	0.78	1.13–4.18			
	Second	30	0.66	0.48	−0.29–1.60	2	0.001	**
	Third	30	−0.05	0.45	−0.92–0.83			

Mixed-effects logistic regression done with lme4 package. Variables include age (numeric variable), breed (categorical variable with two levels), treatment (categorical variable with two levels) and trial (categorical variable with three levels). The results are on the logit scale and estimated marginal means have been calculated with emmeans package for each variable: n, number of observations; EMM, Estimated Marginal Mean; $SE_{(EMM)}$, standard error of EMM; Asymp. 95% $CI_{(EMM)}$, asymptotic confidence interval of EMM; Df, degrees of freedom and p-values were calculated in ANOVA with car package.
*$p < 0.05$; **$p < 0.01$. ns, not significant.

TABLE 2 Summary of the mixed-effects logistic regression on success of solving detour task through use of demonstration route.

Variable	Levels	n	EMM	$SE_{(EMM)}$	Asymp. 95% $CI_{(EMM)}$	Df	*p*	
Age	Continuous	30	−0.27	0.35	−0.95–0.41	1	0.13	ns
Breed	Holstein	19	−0.13	0.42	−0.95–0.68	1	0.60	ns
	Red	11	−0.40	0.57	−1.51–0.70			
Treatment	Observer	15	−0.46	0.49	−1.42–0.49	1	0.56	ns
	Control	15	−0.07	0.47	−1.00–0.85			
Trial	First	30	0.16	0.49	−0.80–1.13			
	Second	30	0.39	0.50	−1.36–0.58	2	0.47	ns
	Third	30	−0.58	0.50	−1.56–0.41			

Mixed-effects logistic regression done with lme4 package. Variables include age (numeric variable), breed (categorical variable with two levels), treatment (categorical variable with two levels) and trial (categorical variable with three levels). The results are on the logit scale and estimated marginal means have been calculated with emmeans package for each variable: n, number of observations; EMM, Estimated Marginal Mean; $SE_{(EMM)}$, standard error of EMM; Asymp. 95% $CI_{(EMM)}$, asymptotic confidence interval of EMM; Df, degrees of freedom and *p*-values were calculated in ANOVA with car package.
ns, not significant.

no significant difference in concordance between observers and controls (estimate $\pm$ SE $= 0.60 \pm 0.90$, $z = 0.67$, $p = 0.50$).

Discussion

This study aimed to investigate if dairy cows possess the cognitive abilities to acquire new behavior through intra-species social learning in a spatial detour task. It was hypothesized that cows observing a trained demonstrator would be more successful in solving the detour task and do so with shorter latencies compared to control cows. Contrary to what was expected, the results showed no significant differences in success between treatment groups. Instead, age appeared to be the most influencing factor. Moreover, the results showed no effect of treatment on the choice of route, i.e., observer cows did not favor the demonstration route. When only considering trials with successful outcomes (i.e., cows that reached the reward bucket within 90 s regardless of route), the latencies of cows choosing the demonstrated route were significantly shorter than the latencies of those choosing the opposite route, even though the routes provided an equal distance to the reward bucket. Moreover, successful observers had a tendency to be faster than successful controls, which could indicate some effect of treatment on cows learning the route. An alternative explanation could, however, be that the presence of the demonstrator in the demonstrator pen might have negatively affected the latencies of cows choosing the opposite route, on which they walked next to the demonstrator pen. Nonetheless, control cows might have been slower since they relied solely on individual learning mechanisms, which could have affected their latency to solve the detour task.

Individual cows with comparable trial outcomes were generally consistent in their choice of route to detour. This is in contrast to the findings of a lack of consistency in the individual detour behavior of goats (23) and adult sheep (43), but in line with some previous findings of detour behavior in horses (25). Furthermore, observers spent a greater proportion of the time before each trial facing the test arena than controls did, meaning they were likely to have seen the demonstration.

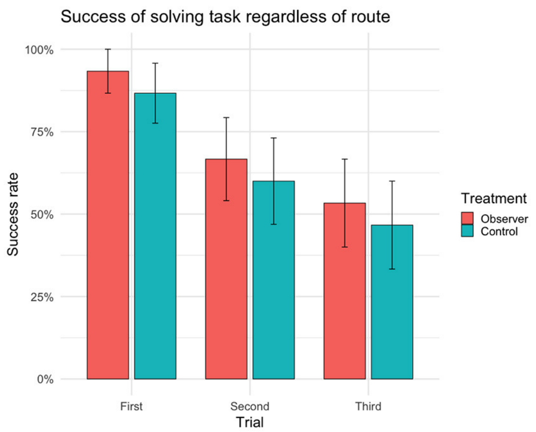

FIGURE 2
Success rate (sample mean) of reaching the yellow reward bucket and solving task within 90 s regardless of route (i.e. achieving complete or partial success) for each trial ($n = 30$ cows, 90 trials). Error bars indicating SEM.

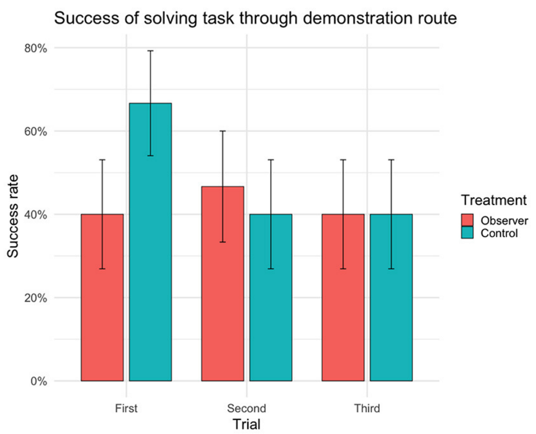

FIGURE 3
Success rate (sample mean) of reaching the yellow reward bucket and solving task within 90 s through demonstration route (i.e. achieving complete success) for each trial ($n = 30$ cows, 90 trials). Error bars indicating SEM.

Collectively, the results of the study indicate that cows did not utilize social learning mechanisms when solving the applied spatial detour task.

Motivation

The overall success rate in first trial was high for both observers and controls, with 27 of 30 cows successfully solving the task within 90 s. Surprisingly, for both treatment groups, latency increased over the following trials. This is in contrast with some of the results for horses, who conversely became increasingly faster over trials (25). As latencies increased and cows failed to reach the bucket within 90 s, the previously high success rate decreased. As most cows managed to reach the reward bucket during their first attempt, it seems that the failure to repeat this success in following trials is more likely to be a reflection of a lack of motivation than an inability to solve the task.

Although all cows seemed motivated to obtain food rewards during habituation and training (e.g., consistently seeking out and emptying buckets, as well as fulfilling the habituation criterion), one of the main challenges during the test was keeping both the test subjects and demonstrator cows motivated throughout repeated demonstrations and trials. Several factors could be at play. The test was performed in the hours between morning and afternoon (from 09:00 to 15:00 h) when the cows normally would be out on pasture, grazing and ruminating/resting. Anecdotally, grazing in the test arena increased during the second and third trial (compared to first trial), and cows would graze on all sides of the obstacle before approaching the reward bucket. Furthermore, some cows would successfully detour the obstacle without approaching the bucket and thus rendering the trial unsuccessful. The closer to the afternoon milking, the more difficult it became to drive the cows from the cowshed to the test arena, indicating a strong motivation to remain indoors. The weather during the test days was generally warmer than during training, with temperatures reaching up to 30°C, clear blue skies, and no wind. This is considered very warm for Swedish summer. The heatwave also brought on an increase in both regular flies (*Musca spp.*) and biting giant horseflies (*Tabanidae spp.*), and the insect harassment clearly affected both test subjects and demonstrator cows. To increase motivation, future studies could benefit from using heifers or dry cows placed under a limited feed regime, as opposed to milking cows with access to plentiful amounts of the same type of concentrates as the food reward, or potentially using a higher-value food reward. Testing in an indoor setting could provide a more controlled environment and eliminate grazing opportunities during the test.

Age

Younger cows were more successful compared to older cows in overall solving the detour task and reaching the yellow reward bucket, however, they were not faster in doing so. Moreover, the effect of age on success does not imply that they were learning from the demonstrator as there was still no effect of treatment on success or the choice of route. Furthermore, as this effect of age was insignificant in first trial where the overall success rate

TABLE 3 Summary of the mixed-effects linear regression on latency to reach reward bucket in successful trials.

Variable	Levels	n	EMM	$SE_{(EMM)}$	95% $CI_{(EMM)}$	Df	p	
Age	Continuous	29	47.1	3.7	39.5–54.8	1	0.97	ns
Breed	Holstein	18	45.7	4.4	36.7–54.7	1	0.69	ns
	Red	11	48.6	5.9	36.4–60.7			
Treatment	Observer	14	41.3	4.9	31.2–51.3	1	0.08	ns
	Control	15	53.0	5.2	42.4–63.7			
Trial	First	27	44.5	4.5	35.6–53.5			
	Second	19	42.3	5.2	31.9–52.8	2	0.15	ns
	Third	15	54.6	5.9	42.7–66.4			
Route	Demonstration	41	39.2	4.2	30.7–47.7	1	0.01	**
	Opposite	20	55.1	5.5	44.0–66.1			

Mixed-effects linear regression done with lme4 package. Variables include age (numeric variable), breed (categorical variable with two levels), treatment (categorical variable with two levels), trial (categorical variable with three levels) and route (categorical variable with two levels). The results are on the response scale and estimated marginal means have been calculated with emmeans package for each variable: n, number of observations; EMM, Estimated Marginal Mean; $SE_{(EMM)}$, standard error of EMM; 95% $CI_{(EMM)}$, confidence interval of EMM; Df, degrees of freedom and p-values were calculated in ANOVA with car package.
$^{**}p < 0.01$. ns, not significant.

FIGURE 4
Latency (sample mean) of reaching the yellow reward bucket and solving task in successful trials ($n = 29$ cows, 61 trials).

was highest, it is likely a reflection of a decrease in the motivation of older cows during following trials.

Motivation toward the acquisition of novel information can be defined as curiosity (44). In young horses, the motivation to explore novel objects (i.e., the level of curiosity) has been shown to be positively associated with learning performance in tests based on both positive and negative reinforcement (45). More research is needed to determine if younger cows are more curious than older cows, and if so, how curiosity acts to motivate the acquisition of novel information. The effect of age on success in this study further underlines the benefits of using heifers in future studies.

Demonstration

As mentioned, the dominance relationship between demonstrator and observer has been suggested to be important for the facilitation of social learning (30). This may be because lower-ranking animals are more attentive toward higher-ranking animals to avoid aggression, or because higher-ranking animals display better fitness and therefore are more attractive to learn from (1, 22). Older cows are more likely to be dominant (46), but as suggested by McVey et al. (25), leadership status may also affect demonstrator significance in a social learning context, and different types of leadership may be important depending on the task. In a spatial detour task, the most relevant leader may be the one who can successfully initiate movement of one or more followers. In this herd, the demonstrators stood out as (i) being initiators of movement, i.e., showing leadership (47), (ii) displaying low fear and high curiosity of the experimenters, which was considered crucial for training, and (iii) winning agonistic encounters with other herd members, i.e., indicating a level of dominance (31, 32). However, these cows were only 3 years old in a group where, at the time of testing, age ranged from 2 to 9 years with a mean of 3.7 years. As research shows that age is likely to play a role in dominance (46) and thus also in social learning (1, 30), future studies may benefit from a more thorough investigation into the social hierarchy (32, 48) and different leaders (47) of the herd before selecting demonstrators.

One of the components of actual social learning is goal emulation (6). To ensure that the observer cows solved the detour task with the goal of accessing the food reward, and not simply as a result of seeking social companionship and thus the proximity of the demonstrator cow, the demonstrator was removed from the test arena after each demonstration. However, as isolation has been shown to increase stress and negatively impact learning and performance in cattle (49), the demonstrator was placed in an adjacent demonstrator pen for social buffering. This demonstrator pen was located next to the obstacle (as opposed to next to the starting pen and the observer) due to concern that the test subjects otherwise might be more inclined to remain close to the demonstrator pen than to solve the task. As the presence of a conspecific has shown to impact detour strategy (25), the demonstration route was placed on the opposite side of the obstacle. Thus, avoiding any potential effect of the demonstrator's presence on the observer's choice of route being mistaken for an effect of social learning from the demonstration. This meant that the demonstrations began with the demonstrator walking toward the observer before rounding the obstacle and proceeding in demonstrating the way to the food reward. It seems likely that this might add another layer of complexity to the demonstration, in terms of both visual and olfactory cues, for the observer to interpret. Future studies featuring a similar task should consider a design that allows for a demonstration starting point that is closer to that of the observer.

An ideal demonstration would have included the demonstrator cow performing the demonstrations independently (i.e., without the human experimenter) and in the same manner in each trial and for each observer cow. This was not achievable and, therefore, the human experimenter had to lead the demonstrator (using the black bucket) to ensure conformity between demonstrations. Hence, had the results of this study shown an effect of demonstration that was indicative of social learning, it could have been discussed whether this effect was evidence of intra-species or inter-species social learning. Future research should ideally design detour tasks that allow for conspecific demonstration of the task, and when not possible, movements of the human experimenters, both before, during and in between trials, should be taken into careful consideration.

Social transmission

Surprisingly, when looking at first trial, the controls appeared to favor the demonstration route, and the observers the route next to the demonstrator pen. Although this effect was insignificant, this raises some questions about the potential role of social transmission. One explanation for the observers favoring this route (while the controls did not) could be that the demonstrator was still chewing on the food from her bowl when the trial started, which may have served as a stimulus enhancement. On the other hand, the demonstrators were observed grazing and chewing on grass throughout the test and in both control and observer trials. Another, perhaps more plausible, explanation could be that watching the demonstrator exit the test arena after each demonstration served as a stimulus enhancement toward the demonstrator pen and potentially the social companionship of the demonstrator cow. This could mean that cows may be equally or more motivated by social companionship than by the food reward used in this study, or it could mean that they interpreted the demonstrator pen as the way out of the test arena and into the pasture.

The difference in choice of route between observers and controls evened out over trials. Regardless, clearer results may be achieved by controlling for social transmission. One alternative could be to implement double control groups; one group where the cows can observe the demonstrator eating from the reward bucket behind the cattle gates before exiting to the demonstrator pen (i.e., partial demonstration), and one group kept as an absolute control (i.e., no demonstration). This would likely need to be compensated with an increase in demonstrators and/or test days, to ensure that the demonstrator cows' motivation doesn't further decrease.

Practical implications

The results of this study add to the sparse body of knowledge of social learning in livestock ungulates. It further serves as a starting point for future research on the cognitive mechanisms utilized by cows faced with spatial problem-solving in a social context. As all but three cows successfully solved the detour task in the first trial, with a lack of effect of treatment on both the overall success of solving the task and on doing so by use of the demonstration route, it seems plausible that most cows learned how to navigate around the obstacle through individual associative learning.

The lack of evidence of social learning does not necessarily mean that these cognitive mechanisms are absent in cattle. It is possible that social learning is not the primary strategy for acquiring new behavior in this specific situation and that the design of this detour task was too simple for there to be a detectable effect of any secondary strategies. Similar results for horses were found by McVey et al. (25), who pointed to the possibility that social learning might be reserved for when individual learning is ineffective. There is also the possibility that the observers did not fully understand the demonstration and that individual learning thus was employed as a secondary, rather than a primary, strategy for solving the task. A spatial problem-solving task with an increased degree of difficulty (and thus a higher risk of failing through individual learning), and a design that allows for a less complex demonstration, could provide a clearer result of the occurrence of social learning in cattle.

The strategy for acquiring new behavior could also be specific to the situation, meaning that cattle may have the ability to utilize social learning in other situations that are unrelated to spatial problems. As studies have shown that lambs can learn to feed select, and thereby avoid poisonous plants, from grazing with their dams (9, 10), it is possible that such risk-reducing foraging strategies (11) could also be utilized by calves grazing with their dams. However, it is also possible that the adaptive value of social learning, at least between peers, is relatively low for grazing cattle in comparison to predator species with more complex foraging behavior. As such, it may be that simpler processes of information transfer (i.e., social transmission mechanisms) have provided enough evolutionary advantages to cattle through, e.g., social facilitation of synchronized behaviors (50–52), feed locations (13), how to graze (7, 8), and the appropriate response to a frightening stimulus (16).

Learning more about the cognition of cattle is important in several aspects, including cattle welfare and the sustainability of the meat and dairy industry. Assumptions of cattle's ability to emulate or imitate the behavior of others (during e.g., moving of animals to new pastures, loading for transport, etc.) can potentially lead to frustration in livestock handlers (53), and rough handling of cows failing to meet these expectations (54). Such handling has negative welfare implications for the individual cow, with the handling-induced stress and fear also leading to production losses (55), and an increased risk for animal-related injuries to livestock handlers (56–58). As such, a deeper understanding of the cognition of cattle may help in the development of housing systems and management routines and has the potential to improve cattle welfare as well as handler safety, while avoiding unnecessary production losses.

Conclusion

The results of this study indicate that cows did not rely on social learning mechanisms when solving the applied spatial detour task. Instead, it seems plausible that most cows learned to solve the detour task through individual learning. More research is needed to determine if this was because cattle do not possess the cognitive mechanisms necessary for social learning or if, in this specific situation, cows primarily utilized other strategies for acquiring novel behavior. Designing a detour task with an increased degree of difficulty that also allows for a less complex demonstration may, together with the implementation of a control for social transmission, provide clearer results. As age and motivation appeared to play a role in this study, future studies could benefit from using older demonstrators, younger test subjects, test subjects placed under a limited feed regime, and/or from using a higher-value food reward.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Board for Animals in Research and Teaching, SLU, Uppsala, Sweden. Written informed consent for participation was not obtained from the owners because oral informed consent was obtained prior to the experiments.

Author contributions

MR applied for and was later awarded funding for the study. JS brought the idea for the experiment. MR, JS, and JY collaborated on finetuning the test adapted from previous work done by MR. MR, JS, and JY participated in the training, habituation, and preparations of the experiment. JS and JY executed the experiment. MR and JS were in charge of data retrieval and editing and performed the statistical analysis together. JY and HB contributed to the interpretation of the results. JS wrote the first draft of the paper under supervision of MR. All authors provided critical feedback and contributed to proof reading and fine-tuning the paper for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Benchmarking calf health: Assessment tools for dairy herd health consultancy based on reference values from 730 German dairies with respect to seasonal, farm type, and herd size effects

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Good calf health is crucial for a successfully operating farm business and animal welfare on dairy farms. To evaluate calf health on farms and to identify potential problem areas, benchmarking tools can be used by farmers, herd managers, veterinarians, and other advisory persons in the field. However, for calves, benchmarking tools are not yet widely established in practice. This study provides hands-on application for on-farm benchmarking of calf health. Reference values were generated from a large dataset of the "PraeRi" study, including 730 dairy farms with a total of 13,658 examined preweaned dairy calves. At herd level, omphalitis (O, median 15.9%) was the most common disorder, followed by diarrhea (D, 15.4%) and respiratory disease (RD, 2.9%). Abnormal weight bearing (AWB) was rarely detected (median, 0.0%). Calves with symptoms of more than one disorder at the same time (multimorbidity, M) were observed with a prevalence of 2.3%. The enrolled farms varied in herd size, farm operating systems, and management practices and thus represented a wide diversity in dairy farming, enabling a comparison with similar managed farms in Germany and beyond. To ensure comparability of the data in practice, the reference values were calculated for the whole data set, clustered according to farm size (1–40 dairy cows ($n = 130$), 41–60 dairy cows ($n = 99$), 61–120 dairy cows ($n = 180$), 121–240 dairy cows ($n = 119$)

and farms with more than 240 dairy cows ($n = 138$), farm operating systems (conventional ($n = 666$), organic ($n = 64$)) and month of the year of the farm visit. There was a slight tendency for smaller farms to have a lower prevalence of disorders. A statistically significant herd-size effect was detected for RD ($p = 0.008$) and D ($p < 0.001$). For practical application of these reference values, tables, diagrams, and an Excel® (Microsoft®) based calf health calculator were developed as tools for on-farm benchmarking (https://doi.org/10.6084/m9.figshare.c.6172753). In addition, this study provides a detailed description of the colostrum, feeding and housing management of preweaned calves in German dairy farms of different herd sizes and farm type (e.g., conventional and organic).

KEYWORDS

diarrhea, bovine respiratory disease, omphalitis, organic farming, benchmarking tool, animal wellbeing and welfare, calf disease

Introduction

The most common disorders in preweaned dairy calves are diarrhea, respiratory disease, and omphalitis (1). Diseases in calves have a variety of negative effects such as growth retardation, a higher susceptibility to develop further diseases and an increased risk of mortality (2–5). A wide spectrum of risk factors affecting calf health have been reported, including energy supply of the dam (6), colostrum supply of the neonate (7), housing conditions (8), and plane of nutrition (9). Previous studies have shown that farm-specific characteristics, e.g., colostrum management (10) and housing conditions (11), are closely related to region and herd size. Season (12), climate (13, 14), number of dairy cows (15), farm type [organic, conventional, (16)], and region also have a great impact.

The health status of its youngstock substantially contributes to the profitability of a dairy farm. Therefore, on-farm monitoring of health indicators should form an integral part of the routine work on dairy farms. Currently, the choice of appropriate health indicators, and the classification of the results obtained with respect to the quality indicate no uniformity among the persons involved in the calf rearing process. Likewise, there is a degree of farm blindness regarding poor conditions (17). For this reason, objective assessment parameters are needed to evaluate the health status of preweaned dairy calves during the farm visit. Benchmarking is a simple established method initially used in industry for comparing the performance of producers with respect to product quality and has been introduced in dairy farming. Benchmarking enables a comparison of farms sharing similar characteristics and simultaneously helps to identify areas for potential improvement (18, 19).

In modern dairy farming, a wide range of sensor data from lactating dairy cows, e.g., milk yield and udder health indicators (20) or indicators of fertility (21), are already systematically collected and analyzed in the daily work routine. Previous studies have already shown that farmers who have access to data related to calf health from other farms are highly motivated to improve their own management practices, e.g., by aiming at increasing average daily weight gains of their preweaned dairy calves (22, 23). In addition, the use of benchmarks can help to reinforce the relationship between farmers and veterinarians (24). In this context, it was also found that farmers motivated by a trusted advisor were more likely to make changes in disease prevention management (25).

Despite the already observed positive effects of benchmarking, to date, there are no widely established hands-on applications available to assess the health status of preweaned calves on farms. There is also a lack of reference values on herd-level for prevalence data on diseases in preweaned dairy calves based on a large study population. The scarce available literature on calf health on organic farms does not yet provide representative data that can be made applicable to all organic farms (26). Furthermore, most studies focus on conventional farms. Therefore, the aim of the present study was to use a large and diverse data set, including 730 German dairy farms and 13,658 examined preweaned dairy calves to provide representative herd level reference values in tables and figures for use in on-farm consultancy. A further aim was to develop a digital calf health calculator which allows farmers, herd managers, veterinarians, and other advisory persons to benchmark farm data on the basis of these reference values.

Materials and methods

Data set

As part of the prevalence study "PraeRi" (25), 731 farms in three regions of Germany with intensive dairy farming were visited on a single occasion between December 2016 and July

TABLE 1 Study population and farm data for 730 dairy farms in Germany stratified by herd size and farm type.

No. farms (*n*)	Median (IQR)[#]							
		Conventional						
	Total ($n = 730$)	Overall ($n = 666$)	1–40* ($n = 130$)	41–60* ($n = 99$)	61–120* ($n = 180$)	121–240* ($n = 119$)	>241* ($n = 138$)	Organic ($n = 64$)
Study population								
Dairy cows (*n*)	84 (44, 88)	90 (48, 206)	27 (22, 34)	51 (46, 57)	85 (69, 102)	162 (137, 204)	426 (317, 644)	42 (27, 80)
Preweaned calves (*n*)	13 (7, 27)	15 (8, 31)	5 (3, 8)	10 (7, 13)	13 (9, 18)	24 (17, 31)	66 (45, 90)	7 (3, 12)
Examined calves (*n*)	12 (6, 25)	13 (7, 28)	5 (3, 7)	9 (6, 12)	12 (8, 17)	21 (16, 29)	41 (37, 71)	6 (3, 11)
Examined calves (%)	96 (83, 100)	95 (82, 100)	100 (100, 100)	100 (86, 100)	100 (89, 100)	94 (87, 100)	79 (64, 89)	100 (89, 100)
Age at weaning (wk)	11 (10, 12)	11 (9, 12)	11 (9, 12)	12 (10, 12)	11 (9, 12)	11 (9, 12)	10 (10, 12)	12 (12, 14)
Area under cultivation								
Total area (ha)	100 (52, 328)	104 (55, 400)	32 (23, 50)	56 (43, 90)	92 (68, 120)	190 (130, 438)	1,300 (726, 2,000)	63 (36, 128)
Thereof grassland (ha)	44 (24, 100)	45 (24, 100)	18(12, 29)	25 (20, 35)	41(30, 64)	78 (50, 130)	280 (128, 455)	40 (26, 63)
Thereof arable (ha)	50 (19, 200)	55 (21, 260)	10 (3, 25)	30 (15, 50)	45 (66, 325)	140 (50, 130)	960 (487, 1,500)	24 (0, 78)

[#]Interquartile range.
*Number of dairy cows.

2019. Farm visits included seven federal states: region north: Schleswig-Holstein ($n = 64$), Lower Saxony ($n = 173$); region east: Mecklenburg-Western Pomerania ($n = 65$), Brandenburg ($n = 65$), Thuringia ($n = 46$), Saxony-Anhalt ($n = 71$); region south: Bavaria ($n = 247$). The final data set included 730 farms with a total of 13,658 calves (one farm with one calf was excluded because information about fecal consistency was missing).

Farm selection

In Bavaria, the farms were randomly selected by a neutral auditing organization for Bavarian dairy farms (Milchprüfring Bayern e.V.) and in the remaining federal states, the farms were randomly selected from the complete list of cattle owners in the Identification and Registration of Bovine Animals in accordance with Regulation (EC) No. 1760/2000 Germany (Herkunftssicherung- und Informationssystem für Tiere, HI-Tier). The selection was made using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). The farms were officially invited by the local authorities or the Dairy Herd Improvement Association. The participation was voluntary. The participation rate varied among the three regions between 6 and 9%. In total, 8,944 farms were invited, and of these, 765 farms were visited. On the day of the farm visit, 731 farms kept preweaned dairy calves [details published elsewhere (1)].

Training of veterinarians and interobserver comparison

The farm visits and clinical examination were performed by 21 veterinarians. The study veterinarians were employed exclusively for this study. A training session lasting several days was conducted before the study started and Standard Operation Procedures (SOP) were defined for the collection and analysis of the data. An interobserver comparison was performed once a year to ensure the quality of the collected data. Each observer received an individual evaluation of his/her achievements. When significant deviations were observed for individual observers, an individual problem analysis was performed [details concerning the Interobserver Reliability are published elsewhere (1)].

Study population

The study population included calves, which received milk or milk replacer, aged 24 h to a maximum of 6 months. In total, a median of 14 (Min: 1; Max: 350) calves were present on the farms at the day of the visit (Supplementary Table 3). Depending on herd size, at least one to a maximum of 75 preweaned calves were clinically examined (Supplementary Table 3). In total, for almost all preweaned calves (96%, IQR: 83–100%) on farm an examination was performed (Table 1). Each calf was identified by the last five digits of its ear tag. Data on age, sex, and breed were collected from the online data bank HI-Tier (www.hi-tier.de). Overall, the median age of preweaned calves enrolled in the study was 37 (IQR: 16–62, Table 1) days. The most commonly reported breeds were Holstein Friesian (73.4%) and Simmental (12.7%). In total, one quarter of the examined calves were male [data published elsewhere (1)].

Clustering by herd size and farm type

The 730 farms differed markedly in herd size, farm operating systems, and management. Depending on the number of dairy cows, the farms were assigned to one of five different herd size groups: 1–40 dairy cows (*n* = 130), 41–60 dairy cows (*n* = 99), 61–120 dairy cows (*n* = 180), 121–240 dairy cows (*n* = 119), and farms with more than 240 dairy cows (*n* = 138). Farms of different size varied regarding the area under cultivation, additional occupation of the farmers (full- or part-time business) and their use of veterinary herd health management (VHHM) advisory services (Tables 1, 2). Due to the structural differences between organic and conventional farms, a separate description of the organic farms was issued. The organic farms had a median of 41.5 (IQR: 27.0, 79.5) dairy cows, cultivated a median area of 63.0 hectares and were sometimes (18.8%) run as part-time businesses (Table 2).

The description of the farms (herd size, area under cultivation, farm type) in dependence on three regions (north, east, and south) is given in the Supplementary Tables 1, 2.

Calf rearing strategies of farms enrolled in the "PraeRi" study

Colostrum management

On the day of the farm visit, an interview with the farmer (or herd manager) was conducted. Questionnaires were used to collect information on calving area (e.g., maternity pen, pen of lactating cows, pasture), colostrum (quantity, feeding strategy),

TABLE 2 Farm organization and the use of veterinary herd health management (VHHM) advisory services on 730 dairy farms in Germany stratified by herd size and farm type.

	N (%)							
		Conventional						
	Total (*n* = 730)	Overall (*n* = 666)	1–40* (*n* = 130)	41–60* (*n* = 99)	61–120* (*n* = 180)	121–240* (*n* = 119)	>241* (*n* = 138)	Organic (*n* = 64*)
Farm organization								
Full-time business	676 (92.6)	624 (93.8)	90 (69.8)	98 (99.0)	179 (99.4)	119 (100.0)	138 (100.0)	52 (81.2)
Part-time business	54 (7.4)	41 (6.2)	40 (30.8)	1 (1.0)	1 (0.6)	0 (0.0)	0 (0.0)	12 (18.8)
Conventional	661 (90.4)	660 (99.1)	127 (97.7)	97 (98.0)	179 (99.4)	119 (100.0)	138 (100.0)	0 (0.0)
Organic	64 (8.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	64 (100.0)
Transition#	6 (0.8)	6 (0.9)	3 (2.3)	2 (2.0)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)
VHHM								
For dairy cows	399 (54.7)	346 (52.0)	107 (82.3)	66 (66.7)	95 (53.1)	45 (37.8)	33 (23.9)	52 (82.5)
For youngstock	159 (21.8)	153 (23.0)	7 (5.3)	12 (12.2)	24 (13.4)	34 (28.6)	76 (55.1)	6 (9.4)

*Number of dairy cows.
#Farms in process of transition from conventional to organic farming (in the analysis they were evaluated as conventional farms).

TABLE 3 Colostrum management in 730 dairy farms in Germany stratified by herd size and farm type.

	N (%)							
		Conventional						
	Total (*n* = 730)	Overall (*n* = 666)	1–40* (*n* = 130)	41–60* (*n* = 99)	61–120* (*n* = 180)	121–240* (*n* = 119)	>241* (*n* = 138)	Organic (*n* = 64*)
Supply								
Sucking the dam	156 (21.4)	129 (19.4)	12 (9.2)	21 (21.2)	50 (27.8)	32 (26.9)	14 (10.3)	27 (42.2)
Bucket feeding	541 (74.2)	504 (75.9)	118 (90.8)	78 (78.8)	123 (68.3)	81 (68.1)	104 (76.5)	36 (56.2)
Esophageal tube	31 (4.3)	30 (4.5)	0 (0.0)	0 (0.0)	6 (3.3)	6 (5.0)	18 (13.2)	1 (1.6)
Quantity								
Up to 3 liters	397 (68.6)	374 (69.3)	93 (78.2)	59 (75.6)	87 (65.9)	61 (68.5)	74 (60.7)	22 (57.9)
>3 to 4 liters	154 (26.6)	140 (25.9)	25 (20.8)	16 (20.5)	36 (27.3)	25 (28.1)	38 (31.1)	14 (36.8)
>4 liters	28 (4.8)	26 (4.8)	1 (0.8)	3 (3.8)	9 (6.8)	3 (3.4)	10 (8.2)	2 (5.3)

*Number of dairy cows.

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and feeding management (milk or milk replacer, solid feed, water) of preweaned calves up to weaning. The majority of farms (74.2%) fed colostrum with a teat bucket (Table 3). In just over a fifth of the farms (21.4%) calves sucked the dam as colostrum feeding strategy. This mainly concerned farms with 61–120 dairy cows (27.8%) and farms with 121–240 dairy cows (26.9%, Table 3). In more than two-thirds of the farms (68.6%), up to 3 l of colostrum were offered (Table 3). Overall, 26.6% of the farms offered 3–4 l of colostrum (Table 3). More than 4 l were rarely fed (4.8%); the highest proportion of farms offering more than 4 l of colostrum were found in the herd size group with more than 240 dairy cows (8.2%, Table 3).

Feeding management and housing conditions in the first 2 weeks of life

During the first 2 weeks of life, preweaned calves in all herds were predominantly fed whole milk (59.7%), followed by acidified whole milk (15.8%), and milk replacer (15.6%, Table 4). The most common maximum volume offered to calves at that age was 6 l per day (44.5%, Table 4). On one quarter of the farms (25.3%), more than 6–8 l per day were fed per calf. Overall, 15% of the farms offered a volume of more than 16 l per day (Table 4). The majority of farms fed the calves twice daily (23). In the first 2 weeks of life, on conventional farms, it was common that calves were kept in single housing (92.9%, Table 4). Every fifth organic farm (23.8%, Table 4) housed calves in groups.

Feeding management and housing conditions from the 3rd week of life

From the 3rd week of life, milk replacer (49.0%) was the main feed component on conventional farms, followed by whole milk (32.1%), other liquid diets, such as a mix of milk replacer and whole milk or yogurt (10.1%), and acidified whole milk (7.5%, Table 5). Organic farms did not offer milk replacer to their calves. More than 6–8 l per day was the most common volume of liquid diets (36.5%), followed by more than 8–10 l per day (27.1%, Table 5). In this age group, more than 16 l per day were offered less frequently (7.4%, Table 5). The majority of farms fed the calves twice daily (23). From the third week of life, it was more common for all herds to keep preweaned calves in group housing (81.9%, Table 5).

Weaning

Overall, the median age at weaning was 11 (IQR: 10–12) weeks of life. The weaning age varied slightly depending on herd size (Table 1). Farms with 41–60 dairy cows offered liquid feeding for a longer period of time (12 weeks) compared to those with more than 241 dairy cows (10 weeks). On organic farms,

TABLE 4 Feeding management and housing conditions of preweaned dairy calves in the first 2 weeks of life on 730 German dairies stratified by herd size and farm type.

	N (%)								
		Conventional							
	Total (*n* = 730)	Overall (*n* = 666)	1–40* (*n* = 130)	41–60* (*n* = 99)	61–120* (*n* = 180)	121–240* (*n* = 119)	>241* (*n* = 138)	Organic (*n* = 64*)
Feeding management								
Whole milk	436 (59.7)	385 (57.9)	100 (76.9)	57 (57.6)	98 (54.7)	66 (55.5)	64 (46.4)	50 (78.1)
Milk replacer	114 (15.6)	114 (17.1)	9 (6.9)	14 (14.1)	39 (21.8)	24 (20.2)	28 (20.3)	0 (0.0)
Acidified whole milk	115 (15.8)	108 (6.2)	13 (9.9)	16 (16.2)	23 (12.8)	16 (13.4)	40 (29.0)	7 (10.9)
Others+	63 (8.6)	58 (8.7)	8 (6.1)	12 (12.1)	19 (10.6)	13 (10.9)	6 (4.3)	5 (7.8)
Offered volume of liquid diet per day								
<6 l	323 (44.5)	299 (45.0)	67 (51.5)	49 (50.0)	88 (48.9)	51 (42.9)	44 (32.1)	23 (37.7)
>6–8 l	184 (25.3)	169 (25.5)	28 (21.4)	29 (29.6)	39 (21.7)	31 (26.1)	42 (30.7)	15 (24.6)
>8–10 l	82 (11.3)	73 (11.0)	17 (13.0)	7 (7.1)	15 (8.3)	15 (12.6)	19 (13.9)	9 (14.8)
>10–16 l	28 (3.9)	26 (3.9)	5 (3.8)	2 (2.0)	13 (7.2)	4 (3.4)	2 (1.5)	2 (3.3)
>16 l	109 (15.0)	97 (14.6)	13 (9.9)	11 (11.2)	25 (13.9)	18 (15.1)	30 (21.9)	12 (19.7)
Housing								
Single	678 (92.9)	629 (94.4)	123 (94.6)	96 (97.0)	170 (94.4)	111 (93.3)	129 (93.5)	48 (76.2)
Group	52 (7.1)	37 (5.6)	7 (5.3)	3 (3.0)	10 (5.6)	8 (6.7)	9 (6.5)	15 (23.8)

*Number of dairy cows.
+Mix of whole milk and milk replacer, yogurt, 1st week whole milk, 2nd week milk replacer.

TABLE 5 Feeding management and housing conditions of preweaned dairy calves from the 3rd week of life on 730 German dairies stratified by herd size and farm type.

	N (%)							
		Conventional						
	Total (n = 730)	Overall (n = 666)	1–40* (n = 131)	41–60* (n = 99)	61–120* (n = 180)	121–240* (n = 119)	>241* (n = 138)	Organic (n = 64*)
Feeding management								
Whole milk	235 (32.1)	185 (27.8)	66 (50.8)	31(31.3)	48 (26.7)	25 (21.1)	15 (10.9)	49 (76.6)
Milk replacer	358 (49.0)	358 (53.8)	38 (29.2)	41(41.4)	94 (52.2)	80 (67.2)	105 (76.1)	0 (0.0)
Acidified whole milk	55 (7.5)	47 (7.1)	8 (6.2)	11 (11.1)	14 (7.8)	2 (1.7)	12 (8.7)	8 (12.5)
Others+	74 (10.1)	69 (10.4)	17 (13.0)	16 (16.2)	21 (11.7)	10 (8.4)	5 (3.6)	5 (7.8)
Volume of liquid diet per day								
0–6 l	122 (17.1)	115 (17.6)	17 (13.1)	14 (14.4)	44 (25.0)	23 (19.8)	17 (12.5)	7 (11.7)
>6–8 l	261 (36.5)	243 (37.2)	40 (31.0)	41 (35.3)	64 (36.4)	41 (42.3)	57 (41.9)	18 (30.0)
>8–10 l	194 (27.1)	179 (27.4)	38 (29.5)	24 (24.7)	44 (25.0)	37 (31.9)	36 (26.5)	14 (23.3)
>10–16 l	85 (11.9)	74 (11.3)	29 (22.5)	13 (13.4)	17 (9.7)	7 (6.0)	8 (5.9)	11 (18.3)
>16 l	53 (7.4)	43 (6.6)	5 (3.9)	5 (5.2)	7 (4.0)	8 (6.9)	18 (13.2)	10 (16.7)
Housing								
Single	125 (17.1)	115 (17.3)	47 (36.2)	27 (27.3)	25 (14.0)	9 (7.6)	7 (5.1)	10 (15.6)
Group	597 (81.9)	542 (81.6)	81 (62.3)	72 (72.7)	152 (84.9)	108 (90.8)	140 (94.2)	54 (84.4)

*Number of dairy cows.
+Mix of whole milk and milk replacer, yogurt.

the calves were completely weaned at a median age of 12 (IQR 12–14) weeks (Table 1).

Random sample of calves for clinical examination

Up to 73 preweaned calves, all calves on farm were clinical examined. When this number was exceeded, a random sampling was taken [details are published elsewhere (1)]. Nevertheless, in a few cases more than 73 calves were examined by mistake. This resulted in a true maximum of 75 calves being examined per farm. The sample calculation was performed with a prevalence of 40% at a confidence level of 95% with a power of 80% and a precision of ± 5% being expected (1, 25).

Clinical examination and definition of disorders

Overall, a clinical examination by trained veterinarians was performed on a median of 12 (IQR: 6–25) preweaned dairy calves. The number of calves varied according to herd size, with a median of 5 to a median of 41 preweaned calves being examined per farm (Table 1). The clinical examination included auscultation of the lungs, palpation of the external umbilical structures, visual examination of the limbs at rest and in motion, taking the rectal temperature, and visual assessment of the fecal consistency [for a detailed description of the clinical examination, see (1)]. All findings were recorded on a data sheet using a scoring system. Assigning clinical signs to different disorders was based on predefined criteria for pathognomonic symptoms (case definition shown in Table 6). The following disorders were addressed: diarrhea (D), omphalitis (O), abnormal weight bearing (AWB), and respiratory disease (RD).

Calves showing characteristic clinical signs of more than one disorder (e.g., thickening of the umbilical structures, and liquid or soft feces) were classified as multimorbid (multimorbidity, M).

Statistical analysis

All statistical analyses were performed using R version 4.1.3 (R Foundation for Statistical Computing, Vienna). Descriptive tables were created using the tableone R package [version 0.13.0, (27)]. The prevalence of disorders was calculated as the percentage of the number of examined sick calves to the number of all examined calves on farm. Reference values for farm-level prevalences were calculated as 10, 25, 50 (median), 75, and 90% quantiles. Two repeated ANOVA measurements were taken to calculate the p-values for the effect of farm size and organic management on the prevalence. For both models, a random effect for region was included to account for clustering. Due to large differences in farm size between organic and conventionally managed farms, the organic management model was additionally adjusted for farm size. A p-value ≤ 0.05 was considered significant.

To account for the higher variability in prevalence on smaller farms, we used funnel plots. These show the confidence interval around the average prevalence for a given number of observations (i.e., number of calves) on the farm (28). Since confidence intervals are wider on farms with a lower number of observations (i.e., calves), this addresses the inherently higher variation in the measured prevalence in smaller farms. The confidence intervals were calculated using the modified Jeffreys method, which are equally tailed and provide better coverage close to 0 and 100% (29). Confidence intervals were calculated for 95 and 99.9% confidence levels and both upper and lower limits were plotted.

Development of the calf health calculator

To calculate reference values for the prevalence at 10, 25, 50, 75, and 90% quantiles based on the number of calves, farm type (organic, conventional), and season, quantile non-parametric additive models were used [R package qgam version 1.3.4, (30)]. The seasonal effect was modeled as a circular spline based on the day of year. The effect of the number of calves was modeled as a restricted cubic spline to account for the higher variability in the upper quantiles (75 and 90%) of prevalence for smaller sample sizes (see funnel plot). The estimated model formed the basis for a spreadsheet using Microsoft® Excel® to allow stand-alone and offline on-farm usage of the farm-specific reference data.

Results

Overall prevalence of disorders on herd level independent of farm type (organic and conventional farms)

The overall prevalence of disorders presented in Table 7 can be used as general reference values in the daily counseling practice. The estimation of the prevalence of the following disorders was conducted for 730 dairies with a total of 13,658 preweaned dairy calves. At herd level, omphalitis (O, median 15.9%) was the most common disorder, followed by diarrhea (D, 15.4%) and respiratory disease (RD, 2.9%). Abnormal weight bearing (AWB) was rarely detected (median, 0.0%). Calves with symptoms of more than one disorder at the same time (multimorbidity, M) were observed with a median herd level prevalence of 2.3%. In these multimorbid calves, disease combinations of O, D, and RD occurred most frequently.

TABLE 6 Case definition of disorders based on characteristic clinical signs detected in the clinical examination.

Clinical examination	Characteristic clinical sign*		Disorder
Visual examination of the limbs at rest and in movement	Unequal load of at least one limb **OR** congenital contracture of the flexor tendons	+/− Other findings	Abnormal weight bearing **(AWB)**
Auscultation of the lungs	Increased, louder breathing sounds	+ Fever - Liquid or soft feces +/− Other findings	
	Reduced, low to complete absence of normal breathing sounds ("silent lung")	+/− Other findings	
	Additional sounds besides normal breathing sounds including crackles or wheezes	+/− Other findings	Respiratory disease **(RD)**
	Reinforcement of the tracheobronchial breathing; breathing sounds that in healthy calves are only heard over the large airways (e. g. the trachea) can be heard over the chest wall	+/− Other findings	
Palpation of external umbilical structures	Inflammatory navel abnormalities: thickening and/or swelling and/or pain and/or heat, excluded uncomplicated umbilical hernia	+/− Other findings	Omphalitis **(O)**
Determination of fecal consistency (directly from rectum)	Feces watery or soupy (runs through fingers)	+/− Other findings	Diarrhea **(D)**
Measurement of transrectal body temperature	>39.5°C[a] <38.0°C[b]	Evaluation only in combination with other clinical signs	

*The presence of the clinical sign is sufficient for diagnosis; +/− clinical sign may be present but does not have to be present; − clinical sign must not be present for diagnosis; + clinical sign must be present for diagnosis.
[a]Defined as fever.
[b]Defined as low body temperature.

Prevalence of disorders on conventional farms stratified by the number of dairy cows

There was a noticeable trend that with an increasing number of dairy cows in a herd, the prevalence of diarrhea (D), omphalitis (O), and multimorbidity (M) also increased. This concerned especially farms with more than 41 dairy cows. On farms with more than 61 dairy cows, the prevalence level of respiratory disease (RD) partly decreased with increasing herd size. The *p*-values for the effect of farm size were calculated as follows: for D ($p < 0.001$), RD ($p = 0.008$), AWB ($p = 0.651$), O ($p = 0.135$), and M ($p = 0.098$). Due to differences between herds in the prevalence of disorders, it was useful to compare farms with similar numbers of dairy cows. The differences in prevalence due to farm size are presented in Tables 8A–E.

A total of 1–40 dairy cows

On farms with 1 to 40 dairy cows ($n = 130$) a minimum of one to a maximum of 19 preweaned calves were examined (median: 5; IQR 3–7). Clinical examination was performed for all preweaned calves (median 100%). On at least 50% of the farms, no calves with disorders were detected (median, 0.0%). In the 75%-quantile of farms, every 4th calf had D (24.3%), every 5th calf had O (20.0%), and every 10th calf had RD (10.8%, Table 8A).

A total of 41–60 dairy cows

On farms with 41 to 60 dairy cows ($n = 109$) all preweaned calves on farm were examined (median 100%; IQR: 86–100%). Clinical examination was conducted for at least one calf to a maximum of 28 calves (median: 9; IQR: 6–12). When considering the median, D was the most common disorder (15.4%), followed by O (12.5%). On at least 50% of the farms, no calves with RD, AWB, and M were observed (median 0.0%). In the 75%-quantile of farms, at least one third of the examined calves had O (33.3%) or D (33.3%) and at least more than 1 of 10 calves suffered from RD (11.1%) or M (11.4%, Table 8B).

A total of 61–120 dairy cows

At least 2 to a maximum of 46 preweaned calves per farm were examined (median: 12; IQR: 8–17) in a herd size with 61–120 dairy cows ($n = 180$). This corresponds to almost all presented preweaned calves on farm (median 100%; IQR: 86–100%). In this herd size group, O was the most common disorder with a median herd level prevalence of 21.2%, followed by D

TABLE 7 Herd prevalence of disorders in 13,658 preweaned calves on 730 German dairies.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease (RD)	0.0	0.0	**2.9**	12.2	21.2	7.8
Diarrhea (D)	0.0	0.0	**15.4**	26.2	37.6	17.1
Abnormal weight bearing (AWB)	0.0	0.0	**0.0**	0.0	1.5	1.0
Omphalitis (O)	0.0	4.3	**15.9**	30.2	50.0	20.6
Multimorbidity (M)*	0.0	0.0	**2.3**	10.0	17.6	6.4
M_RD[*a]	0.0	0.0	**0.0**	2.6	8.3	2.4
M_D[*a]	0.0	0.0	**0.0**	7.7	14.3	4.8
M_AWB[*a]	0.0	0.0	**0.0**	0.0	0.0	0.4
M_O[*a]	0.0	0.0	**0.0**	8.3	16.7	5.4

$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile.
*Calves showing characteristic clinical signs of more than one disorder at the same time.
[a]Each subset of superordinate group Multimorbidity (M) with the occurrence of the following disorder combinations: RD, D, AWB, and O. Bold indicates the median values.

TABLE 8A Herd prevalence of disorders in preweaned dairy calves on 130 farms with 1 to 40 dairy cows*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	0.0	**0.0**	10.8	25.0	6.7
Diarrhea	0.0	0.0	**0.0**	24.3	40.0	12.3
Abnormal weight bearing	0.0	0.0	**0.0**	0.0	0.0	1.4
Omphalitis	0.0	0.0	**0.0**	20.0	50.0	13.5
Multimorbidity	0.0	0.0	**0.0**	0.0	14.3	4.0

*At the day of farm visit with a median of 5 (IQR: 2.5–7.0), calves underwent clinical examination.
$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile. Bold indicates the median values.

(14.9%), and RD (5.7%). On at least 50% of the farms, no calves with more than one disorder at the same time (multimorbidity) were detected (median 0.0%). In the 25%-quantile of the farms, almost 1 of 10 calves had an omphalitis (9.1%, Table 8C).

A total of 121–240 dairy cows

In a herd size between 121 and 240 dairy cows ($n = 119$) at least 3 to a maximum of 63 preweaned calves were examined (median of 21; IQR: 16–29). Of the total number of calves on farm, on median 94% (IQR: 87–100%) of the calves were examined. When considering the median, O was the most common diagnosis (18.1%), followed by D (15.4%) and RD (5.6%). In the 25%-quantile of farms, D and O were detected with a prevalence of 7.9 and 12.5%, respectively. On the 10% of farms with the lowest prevalence, O was found in 7.1% of the examined calves (Table 8D).

A total of 241 and more dairy cows

On farms with more than 241 (Max: 2,821) dairy cows ($n = 138$) at least 5 to a maximum of 75 preweaned calves were examined (median 41; IQR: 37–71). Clinical examination was carried out for 79% (IQR: 64–89%) of the total number of calves on farm. Diarrhea (21.0%) was the most common diagnosis, followed by O (19.5%), and RD (5.3%). In the 25%-quantile of farms, D, O, and RD were detected with a prevalence of 14.9, 12.9, and 2.6%, respectively. Even on the 10% of farms with the lowest prevalence, calves with D (8.6 %), O (9.5%), and M (0.9%) were found (Table 8E).

Prevalence of disorders in calves on organic farms

Table 9 assesses the health status of the preweaned calves on organic farms. The organic farms ($n = 64$) enrolled in this study had a median of 42 (IQR: 27–80; Min: 1; Max: 297) dairy cows. At the day of the farm visit, at least one to a maximum of 33 calves were examined (median: 6; IQR: 3–11). Considering the median (IQR: 89–100%), an examination of all preweaned calves on farm was conducted. The most common calf disorders observed on organic farms were diarrhea (D, 8.7%) and omphalitis (O, 8.5%). On at least 50% of the organic farms, no calves with respiratory disease (RD), abnormal weight bearing (AWB) and multimorbidity (M) were observed. In the 75%-quantile of farms, every 4th calf had D (25.0%) and every 5th calf had O (20.8%). Respiratory disease (4.7%) and M (4.1%) were detected to a lesser extent (Table 9). There was a noticeable tendency for organic farms to have a lower prevalence of disorders than similar sized conventional farms. The farm-size adjusted p-values of this effect were calculated for the following disorders: D ($p = 0.092$), RD ($p = 0.082$), AWB ($p = 0.127$), O ($p = 0.295$), and M ($p = 0.441$). Although a difference in the prevalence of disorders was observed between organic and similar sized conventional farms, however, this was not statistically significant.

Evaluation prevalence adjusted for the number of examined calves (funnel plots)

The funnel plots (Figure 1) visualize the distribution of the prevalences of disorders according to the number of calves on the farm. The number of farms can also be determined from the size of the dots in the diagram. For every possible number of examined calves (up to 75) on a farm, confidence intervals around the overall average prevalence were calculated. The confidence intervals were calculated for 95 and 99.9% confidence intervals. The lower the number of calves, the wider the confidence interval. The confidence intervals can be used to assess whether the disorders occur sporadically, are randomly distributed, or occur at an increased rate. In cases of sporadic occurrence, not more than 1 in 20 farms should lie outside of the interval for 95% CI (yellow marked area) and not more than 1 in 1,000 for 99.9% CI (red marked area). If more farms are outside of the CI, this can be an indication that major outbreaks are common for this particular disease. For the individual farm, this indicates a major outbreak is in progress. For example, it can be observed that diarrhea and omphalitis often lead to larger outbreaks (Figure 1). For abnormal weight bearing, there

TABLE 8B Herd prevalence of disorders in pre-weaned dairy calves on 99 farms with 41–60 dairy cows*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	0.0	**0.0**	11.1	25.6	9.1
Diarrhea	0.0	0.0	**15.4**	33.3	41.8	18.7
Abnormal weight bearing	0.0	0.0	**0.0**	0.0	1.1	0.9
Omphalitis	0.0	0.0	**12.5**	33.3	50.0	19.2
Multimorbidity	0.0	0.0	**0.0**	11.4	20.0	6.5

*At the day of farm visit with a median of 9 (IQR: 6.0–12.0), calves underwent clinical examination.
$Q_{0,1}$: 10%-quantile; $Q_{0,25}$: 25%-quantile; $Q_{0,75}$: 75%-quantile; $Q_{0,9}$: 90%-quantile. Bold indicates the median values.

TABLE 8C Herd prevalence of disorders in pre-weaned dairy calves on 180 farms with 61–120 dairy cows*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	0.0	**5.7**	14.3	23.9	9.0
Diarrhea	0.0	0.0	**14.9**	25.0	35.2	16.4
Abnormal weight bearing (AWB)	0.0	0.0	**0.0**	0.0	0.0	0.9
Omphalitis (O)	0.0	9.1	**21.2**	40.3	60.0	26.1
Multimorbidity	0.0	0.0	**0.0**	12.5	19.0	7.3

*At the day of farm visit with a median of 12.0 (IQR: 8.0–17.0), calves underwent clinical examination.
$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile. Bold indicates the median values.

TABLE 8D Herd prevalence of disorders in pre-weaned dairy calves on 119 farms with 121–240 dairy cows*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	0.0	**5.6**	12.5	19.1	8.2
Diarrhea (D)	0.0	7.9	**15.4**	22.9	36.6	17.3
Abnormal weight bearing (AWB)	0.0	0.0	**0.0**	0.0	3.4	1.3
Omphalitis (O)	7.1	12.5	**18.1**	31.6	52.6	24.0
Multimorbidity	0.0	0.0	**5.3**	10.0	17.4	6.8

*At the day of farm visit with a median of 21 (IQR: 16.0–29.0), calves underwent clinical examination.
$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile. Bold indicates the median values.

TABLE 8E Herd prevalence of disorders in pre-weaned dairy calves on 138 farms with more than 241 dairy cows*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	2.6	**5.3**	10.2	15.4	7.0
Diarrhea (D)	8.6	14.9	**21.0**	29.2	36.0	22.7
Abnormal weight bearing (AWB)	0.0	0.0	**0.0**	0.0	2.5	0.5
Omphalitis (O)	9.5	12.9	**19.5**	26.8	35.0	20.8
Multimorbidity	0.9	2.9	**5.8**	10.5	16.2	7.6

*At the day of farm visit with a median of 41 (IQR: 37.0–71.0), calves underwent clinical examination.
$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile. Bold indicates the median values.

is no increased clustering within farms. Respiratory disease also shows a minor tendency for an increased frequency of occurrence, and multimorbidity results from a combination of all disorders.

Calf health calculator

Prevalence depending on season, farm size, and farm type

Figure 2 shows the estimated quantile functions, which are the basis for the calf health calculator. The seasonal effect was modeled as a circular spline (i.e., after December comes January) based on the day of the year. The number of calves was estimated as a restricted cubic spline and estimated values are given exemplarily ($n = 10$, $n = 20$, $n= 30$, etc.) for conventional and organic farms. The level of prevalence in the 90%-quantile decreased with increasing number of examined calves on the farm. The levels of the other quantiles ($Q_{0.1}$, $Q_{0.25}$, median, $Q_{0.75}$) were not affected by the number of examined calves on the farm. Respiratory diseases, D, and M occurred more frequently in the fall. Omphalitis was most common in the summer months. At the individual animal level, it was already determined that no seasonal effect can be represented for abnormal weight bearing (1). Therefore, in the present study, the prevalence of abnormal weight bearing by season is not illustrated.

Calf health calculator in excel

On the basis of the results obtained in the present study, a digital calculator was developed that allows for classification of the health status of preweaned dairy calves as determined on a farm visit through real-time access by comparison with the underlying study population. Reference

TABLE 9 Herd prevalence of disorders on herd level on 64 organic farms*.

Disorder	$Q_{0.1}$	$Q_{0.25}$	Median	$Q_{0.75}$	$Q_{0.9}$	Mean
Respiratory disease	0.0	0.0	**0.0**	4.7	19.0	5.5
Diarrhea (D)	0.0	0.0	**8.7**	25.0	33.3	14.2
Abnormal weight bearing (AWB)	0.0	0.0	**0.0**	0.0	0.0	0.2
Omphalitis (O)	0.0	0.0	**8.5**	20.8	47.0	14.7
Multimorbidity	0.0	0.0	**0.0**	4.1	14.3	4.9

*At the day of farm visit with a median of 6 (IQR: 3.0–11.0), calves underwent clinical examination.
$Q_{0.1}$: 10%-quantile; $Q_{0.25}$: 25%-quantile; $Q_{0.75}$: 75%-quantile; $Q_{0.9}$: 90%-quantile. Bold indicates the median values.

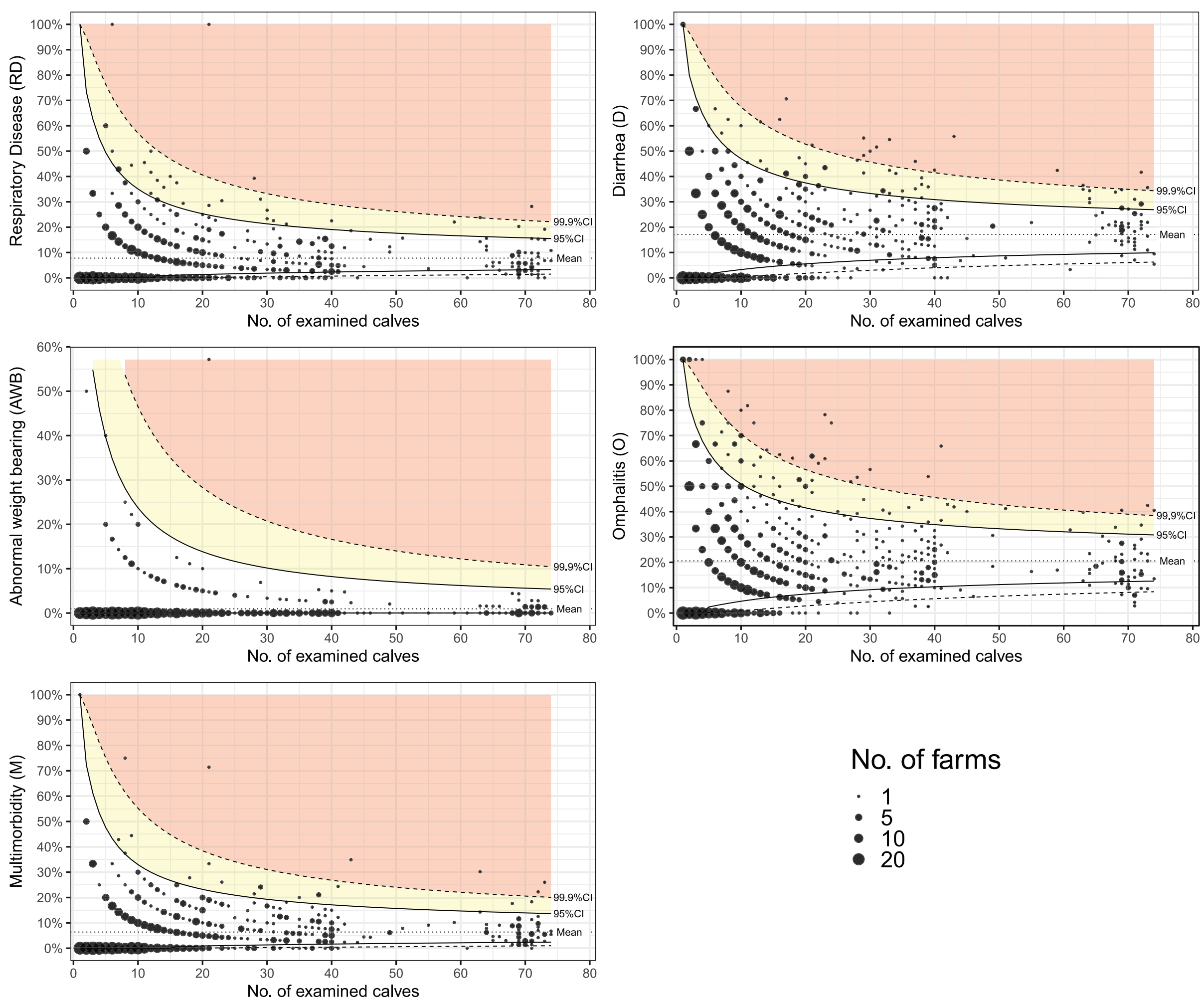

FIGURE 1
Prevalence of disorders stratified by number of examined preweaned dairy calves on the farm. The dotted line marks the mean, the solid line marks the upper and lower 95% CI, and the dashed line marks the upper and lower 99.9% CI. In cases of sporadic occurrence, not more than 1 in 20 farms should lie outside of the interval for 95% CI (yellow marked area) and not more than 1 in 1,000 for 99.9% CI (red marked area). The size of the spots represents the number of farms with the similar prevalence of disorders and number of examined calves. Therefore, there are more farms included, as the size of the dot increases.

values for the disease prevalence for respiratory disease (RD), diarrhea (D), omphalitis (O), and multimorbidity (M) were included. A model was fitted to account for seasonal effects, farm type (organic, conventional) and number of examined calves. As reference values, the 10, 25, 50 (median), 75, and 90% quantiles of the observed data were used. The

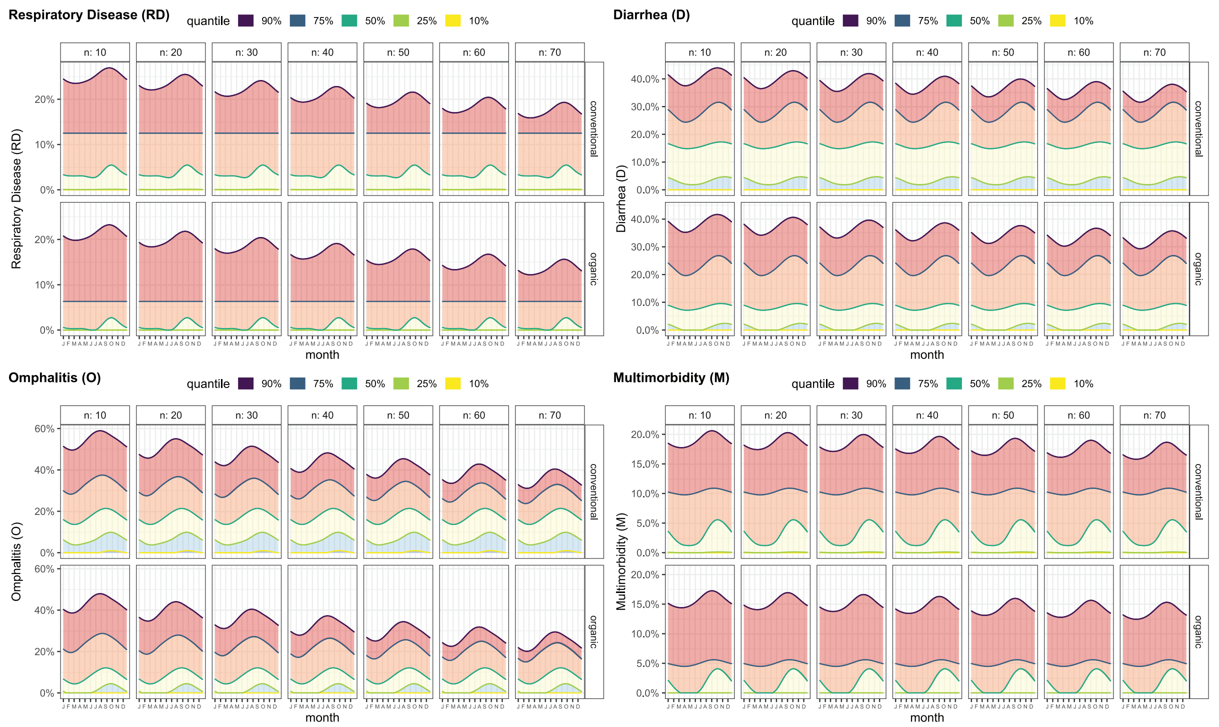

FIGURE 2
Prevalence of disorders based on number of preweaned dairy calves examined at the day of the farm visit for conventional and organic managed dairy farms depending on day of year/month. Quantile functions were estimated using a quantile non-parametric additive model (qgam) for 10, 25, 50, 75, and 90% quantiles. A continuous effect was estimated using restricted splines for number of calves and day of year (circular). Exemplary values are shown for the number of calves ($n = 10$, $n = 20$, $n = 30$, etc.). The farm type (organic, conventional) effect was estimated as a factor. These functions form the basis for the calf health calculator.

estimated reference values were entered into a spreadsheet for on-farm use. The calf health calculator estimates the farm-specific prevalence and benchmarks the results using the reference values based on the underlying study population automatically. The calculator is available as a stand-alone tool for Microsoft® Excel® (https://doi.org/10.6084/m9.figshare.c.6172753).

Discussion

The present study for the first time provides a deep insight into the health status of preweaned dairy calves on herd-level in three different regions of Germany on farms that differ in herd size, management, and farm type (conventional, organic). Data were obtained from databases of the testing associations affiliated with the farms and collected during farm visits on a single occasion by interview, visual observations, and clinical examinations of individual calves. The results proved to be suitable to establish benchmarks that allow for comparisons of the health status of preweaned dairy calves on farms by herd size, farm type, and month of the year. A digital tool ("calf health calculator") was developed that enables farmers, veterinarians and other advisory persons, to classify the health-status of preweaned calves in real-time subsequent to on-farm data acquisition on a farm visit.

Pattern validity of the data set and qualification as reference values

To our knowledge, the "PraeRi" (1, 25) study is the first cross-sectional study presenting such an extensive and diverse dataset including the description of the prevalence of disorders of preweaned dairy calves on German dairies. The final data set included 730 dairy farms with a total of 13,658 preweaned dairy calves. The surveyed farms were located in three different regions of Germany with intensive dairy farming (31) and exhibited marked differences in herd size, structure and management practices (1). These different farms represent a wide variety of production characteristics and thus allow a wide variety of different effects of potential risk factors under field conditions. The farm visits took place continuously in a 3-year period (December 2016 to July 2019). The farms were randomly selected, and regular monitoring of the selection process ensured a high level of certainty in the random sample of farms. The clinical examination of the preweaned calves was performed by 21 trained veterinarians employed explicitly for this study. Trainings for this purpose was conducted and standard operation procedures were developed. In addition, regular meetings and interobserver reliability tests were performed to ensure a high reliability of the recorded data (1). Thus, the estimated prevalences are suitable as reference values for benchmarking dairy farms with a wide range of different herd size, management structures, and geographic location. Due to the diversity of German dairy farming, these data are useful for comparing similarly structured farms in other regions and countries as well.

Alignment of the estimated herd level prevalence with previous studies

In the current study, the overall median herd level prevalence for diarrhea (D, 15.4%) was slightly higher than reported from Chilian dairy farms (15). However, the authors suggest that the mean herd level prevalence of 12.7% may be underestimated due to the small number of examined calves and the fact that the diagnosis was only made by visual inspection (15). In a Canadian study (32), the median within-pen prevalence for D was 17.0% (IQR: 7.0–37.0%). In 19 commercial dairies in Minnesota and Ontario a median herd level incidence risk of 10.5% for D was determined (12). The estimated median herd level prevalence for respiratory disease (RD, 2.9%) corresponds to results from a Norwegian cross-sectional study (33). Other authors, reported noticeable higher prevalence for RD. In a Chilian study, a median herd level prevalence of 17.7% for RD was detected (15) and in an US study 12.0% of the preweaned heifers were affected by RD (34).

Prevalence of omphalitis

The results of this study indicate that omphalitis (O) accounted for the largest proportion of disorders (15.9%) at herd level. The literature is primarily focused on D and RD as the most common disorders in preweaned dairy calves (35–37). In contrast, there is only scarce literature on the prevalence and kind of O on dairy farms primarily focusing on male calves by observations during a limited period of time and under poor conditions (38–41). The diagnosis of O cannot be made on the basis of visual observation alone but requires the collection of vital parameters and palpation of the umbilical region of the calf. Therefore, it is likely that the diagnosis O is missed when monitoring calf health on farms. Analysis of the data at individual animal level demonstrates that O is frequently associated with other disorders such as D, RD, or AWB (1). In addition, omphalitis should also be considered as a possible cause of growth retardation (39), an increased susceptibility to other diseases and mortality (41) in preweaned dairy calves. Our findings show that farmers and veterinarians should devote special attention to the umbilicus at parturition and in the 1st days of life of the neonate. In addition, umbilical disorders should find more consideration in future research.

Prevalence of disorders stratified by herd size

The results of the present study demonstrate clear associations between the herd size and the prevalence of disorders in dairy calves. From the literature, it is well-known that herd size has an effect on the prevalence of calf disorders (42–45). For the effect of farm size, a statistically significant difference for diarrhea and respiratory disease was found. An effect of farm size on omphalitis, abnormal weight bearing, and multimorbidity could also be observed, but this was not statistically significant. Herd size is usually known and therefore enables a comparison between similarly structured farms. Due to the diversity of dairy farming in Germany, the findings of this study can be used for comparison of similarly structured farms of other regions and countries. The proportion of calves on dairy farms affected by the disorders addressed in the present study increased with herd size. On smaller farms, fewer preweaned calves are kept, thus probably reducing the risk of infection, and leaving more time for individual care of calves. However, it should be noted that an effect of herd size could not be found for all studied diseases. Furthermore, as the number of calves examined on smaller farms was lower, the recorded diseases have a more pronounced effect on the herd level prevalence of the farm. Nevertheless, the authors assume as does Kaske (46) that in cases of high prevalence of disorders, there may be deficits in management and hygiene on the farm. Therefore, herd size should be taken into account when including disease prevalence in on-farm benchmarking.

Herd size and omphalitis

The results of this study revealed that with increasing herd size the prevalence of omphalitis (O) increased as well. Omphalitis results from mixed bacterial infections of the umbilical structures (47). From the site of infection, bacteria can spread into single joints or even cause fatal systemic infections (48). It is likely that the higher number of calvings on large dairy farms increase the infection pressure, reduce the time spent on navel disinfection in individual calves or increase the occurrence of navel sucking in larger groups of calves of the same age (49). To prevent O, adequate hygiene in the maternity pen, quick removal of the newborn calves from the maternity pen as well as quick supply of larger volumes of high-quality colostrum (50, 51) are considered crucial. Disinfection of the umbilical structures after birth can also reduce the risk of infection (52).

Herd size and respiratory disease

In farms with a herd size of <60 dairy cows, when considering the median, no calves with respiratory diseases (RD) were detected. The highest prevalence of RD was observed for herd sizes of 61–120 dairy cows (5.7%). With increasing numbers of cows, the prevalence of RD slightly decreased. Housing conditions for the preweaned calves have a great impact on the occurrence of RD. On smaller farms, calves were more often kept in smaller groups (≤7 calves), which could have reduced the occurrence of RD (37, 53). In Germany, on farms with up to 120 dairy cows, it is more common to use old buildings for calf rearing and, in some cases, prophylactic measures are not implemented (26). In unventilated barns, the climatic conditions are often poor. Effects of sudden changes in ambient temperature and humidity, exposure to dust and toxic gases as well as deficient biosecurity measures promote higher prevalences of RD (8, 14). Moreover, on larger farms, it is more established to use prophylactic measures such as a vaccination against RD compared with smaller farms (54). In a Norwegian study it was observed that in larger herds (> 50 dairy cows), the number of animals susceptible to infection was also higher, which can lead to more infections during an outbreak (43). This also promotes the possibility of pathogens circulating within a herd over a longer period and thus can cause infections again and again (55).

Herd size and diarrhea

The highest median herd level prevalence for diarrhea (21.0%) was recorded for farms with more than 240 dairy cows. In this herd size, the prevalence for diarrhea was also highest in the top 10- and 25%-quantile of the farms. Diarrhea is multifactorial by origin including infectious and non-infectious factors. The most common pathogens causing diarrhea in neonatal calves are enterotoxigenic *E. coli* (ETEC), rota- and coronaviruses, and cryptosporidia (56–58). The pathogens are present on every dairy farm (ubiquitous) and the infection happens *via* the environment (maternity pen, housing of calves, teat buckets, etc.) or by contact between calves. However, higher internal infection pressure on farms increases the risk of infection. Higher numbers of calvings and a high stocking density in combination with deficient hygienic conditions lead to an accumulation of pathogens in the animals‘ surroundings increasing the risk of infection (42). The latter is especially true for cryptosporidium that can survive for a long time in the environment (44).

Herd size and multimorbidity

The proportion of multimorbid calves increased with increasing herd size. The occurrence of diseases, especially in the first 2 weeks of life, can promote the development of other diseases (12, 59). The previous disorders may cause immunosuppression and may result in a vulnerability to further diseases (60). Moreover, with increasing herd size the time for health monitoring of the individual calf will decrease (42). This can limit the timely detection and treatment of the disease which can lead to the manifestation of more than one disorder simultaneously.

Calf rearing strategies on organic farms and prevalence of disorders

Due to the clear differences in structure and management of organic and conventional farms, a separate assessment of the health status of the preweaned calves is indicated. Organic farms tended to have fewer dairy cows (median 42) compared to conventional farms. A cross-sectional study in Michigan and Ohio, USA (54) also showed significant differences in management practices between organic and conventional farms. Similar to our observations on conventional farms, it was more common to hand feed the colostrum (304 of 448 farmers), whereas the majority of organic farmers (69 of 171) let the calves suck the dam for colostrum intake. In the same study (54), it was observed that conventional producers separated the calves from the dam earlier after birth compared to organic producers. There is already a study from the United States reporting parameters of the health status of preweaned dairy calves on organic farms. In this study, the incidences for D (44.4%) and RD (11.5%) were significantly higher than the prevalence reported in the current study. Possible reasons for these differences may be that the farms in the US study were not randomly selected, and that the disease recording was not done by veterinarians but by farm personnel (26). To our knowledge, ours is the first representative cross-sectional study reporting the prevalence of disorders in preweaned dairy calves on organic farms. In the current study, there is a noticeable tendency for organic farms to have a lower prevalence of disorders than similar sized conventional farms. However, this effect is not statistically significant. The impact of these or other unrecorded management factors, especially of organic farms on the prevalence of disorders in preweaned dairy calves needs to be clarified in further studies.

Prevalence of disorders depending on season

In the fall and winter, respiratory diseases, diarrhea, and multimorbidity had the highest prevalence. Calves born in the fall had a 1.8- and 2.0-times higher risk of being treated for diarrhea compared to those born in the spring or summer (12). Possible causes of higher disease rates may be a lower colostrum quality in winter (61, 62) and a higher shedding of the pathogens (e.g., cryptosporidium) compared to the summer season (44). The lower temperature and the higher humidity in the fall and winter months provide better survival chances, for example the oocytes of cryptosporidium (63). In contrast, the highest prevalence of omphalitis was detected in the summer. A wide range of opportunistic bacteria are often involved in umbilical infections (46). With increasing temperature, the bacteria in the environment proliferate, which might increase the risk of infection. In the summer months, dust and flies can also act as predisposing factors (64).

Hands-on applications and calf health calculator

The added variability due to a low number of observations is a major problem when comparing farm level prevalence on small farms with reference data. On farms with three calves, the prevalence can only be either 0% (0/3), 33% (1/3), 66% (2/3), or 100% (3/3). Thus, it is quite easy to observe a high prevalence due to random variation. To address this problem, in the present study, funnel plots were used for visualization (62). For every possible number of examined calves (up to 75) on a farm, confidence intervals around the overall average prevalence were calculated. The lower the number of calves, the wider the confidence interval (27). This addresses the problem of the higher variation due to a lower number of observations (i.e., calves). Funnel plots are helpful to easily identify sporadic occurrences or an outbreak of a disease (28). Based on the differences demonstrated for the prevalence of the individual disorders according to the number of calves, month of the farm visit, and the farm type (conventional, organic) these factors build the basis for the calf health calculator. As already discussed in the section on funnel plots (see above), the number of calves was added to account for the higher variability on farms with a lower number of calves, i.e., higher thresholds for small farms. Additionally, a continuous effect based on the day of year was estimated to account for seasonality in disease occurrence. Abnormal weight bearing was omitted because of the very low prevalence (1).

Application in practice

The objective of this study was to transform the estimated prevalence for calf disorders based on a large and diverse data set into an applicable form for use in practice. In herd management of dairy farms, it is already common practice to use health data e.g., chewing activity, rumen fill, and fecal consistency for monitoring the health status of dairy cows. Farmers, herd managers, veterinarians and other advisors use tools based on these data to develop farm-specific concepts and management recommendations. In contrast, there are still no established uniform monitoring measures for calves. A Canadian study discovered that only one third of the veterinarians regularly asked about the health and performance of the calves on routine herd visits; as many as 13% of the surveyed veterinarians never asked about the calves (65). This is particularly problematic, as the consulting veterinarians play a key role in implementing changes in management practices to improve on-farm health (66) and are an important source of information about dairy herd health and management (67). Interviews with farmers revealed that they value communicating with the herd consulting veterinarian about calf health and development, and benchmarking can motivate them to make changes affecting calf management (22). In addition, farmers motivated by a trusted advisor were more likely to make changes in disease prevention management (68). Furthermore, benchmarking can help to reinforce the relationship between farmers and veterinarians (22).

The tools (table, diagrams, and digital calf health calculator) developed in this study will now be available to farmers, herd managers, veterinarians, and other advisors to help include internal or external calf health monitoring in their work routine. By documenting calf health on the farm using the calf health calculator, the authors hope to improve monitoring of calves on the farm, while detecting diseases more quickly and identifying potential problem areas. However, if the recording of the health status of the preweaned calves is carried out by non-trained personnel and in a less standardized way, as done in the present study, it is possible that measurement errors and deviations from the reported reference values may occur. Nevertheless, these differences in recording and classification are consistent within a person, so that this method is still suitable for assessing calf health on the farm. In addition, the documented data enables a permanent controlling and comparing within the farm as well as with other farms. Through the use of benchmarking, the authors expect that calves will become more visible in dairy farms as well as in consultancy practices, which may lead to sustained improvements in calf health.

Due to the size and diversity of the study population, these data allow farmers to compare themselves with similarly sized and structured farms. This high level of identification gives the data much greater credibility in consulting practice than reference values taken from farms that differ clearly in size, structure, and management. An update of the reference values applied in this study will not take place in the near future, because another study with such an extensive data set like the PraeRi study is not yet planned. In order to make these tools available for other study populations, the used code will be provided in the Supplementary material. Furthermore, a translation of the calf health calculator into other languages (currently German and English are available) is planned, as well as the development of a Libre Office version.

Conclusion

At herd level, omphalitis (O) was the most detected disorder. This is particularly interesting because in the literature, diarrhea (D) and respiratory diseases (RD) are discussed as the main causes of calf disorders. Therefore, more attention should be paid to O in future studies and in the practice as well. Moreover, the current study demonstrated marked differences in the prevalence of disorders (D, RD, O, AWB, and M) between herds which partly could be explained by herd size, farm type (organic, conventional), and season. Thus, for a viable benchmarking, it is useful to take these factors into account. Overall, our results reveal that calf health should become a central issue for dairy farmers and in veterinary herd health consultancy. The benchmarks developed in this study should provide a practical tool for assessing on-farm calf health. Due to the extensive and diverse data set of the "PraeRi" study and the diversity of dairy cow farming in Germany, we assume that the results of this study can be transferred to other regions and countries as well.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because no painful interventions were made. This was in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LD, HA, ARB, LK, AnS, KB, PD, MK, PP, AlS, and SW visited the farms and collected the data. LD, AB, HA, and MH analyzed the data. AB, LD, HA, and MH developed the calf health calculator. LD, HA, and AB wrote the first draft of this work and discussed the results with MH. MH, KM, and GK-S acquired the funding for the realization of the project PraeRi. All authors were involved in the planning of the study, revised the article critically, and contributed substantial ideas. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author KB was employed by VetZ GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Companion animal adoption and relinquishment during the COVID-19 pandemic: Peri-pandemic pets at greatest risk of relinquishment

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The COVID-19 pandemic has created a situation globally where companion animals may be at increased risk of relinquishment and abandonment due to multiple interrelated factors. The aims of this study were to establish the prevalence of self-reported adoption and relinquishment of cats and dogs during the pandemic, and to identify characteristics associated with relinquishment. A survey was distributed to 4,000 participants across several countries including the UK, USA, Canada, Italy, Spain and France. N = 3,945 responses were available for analysis. Three groups of participants were identified; Those that never considered relinquishment (NCR), those that have considered relinquishment (CR) and those that have already relinquished a cat or dog (R). Two follow-up surveys were sent to CR and R participants. Considering data from the three surveys, 4.06% of participants considered giving up their pet, 0.74% relinquished their pet, and 0.2% considered and then later give up their pet. Compared to pets given as a gift, there was a 38.7% decreased likelihood of relinquishment in pets sourced from a shelter ($P < 0.001$), 31.2% decrease in those sourced from a breeder, and a 24.4% decrease in those acquired directly from someone that needed to find a new home for their cat or dog. Compared to owners who acquired their pet > 6 months before COVID-19 was declared a pandemic, those acquired < 6 months before COVID-19 was declared a pandemic were three times more likely to be considered for, or be, given up ($P < 0.001$) and those acquired after COVID-19 was declared a pandemic were two times more likely to be considered for, or be, given up ($P < 0.001$). There was a trend for greater likelihood of CR or R of pets acquired online ($P = 0.074$). Of those that had already given up their pet, 14.3% relinquished to a shelter, 66.7% gave their pet to a new owner and 19% obtained temporary care from someone else. A total of 65.0% of CR participants were male, increasing to 72.2% of R participants. There was no effect of species (cat or dog) on risk of relinquishment. Financial constraints were the most mentioned reason for both giving up a pet and considering giving up a pet, followed by health concerns specific to COVID-19, and behavioral problems. The findings from this study should be used to inform interventions aimed at reducing companion animal relinquishment.

KEYWORDS

adoption, relinquishment, cat, dog, COVID-19, online

1. Introduction

During times of crisis, there is an increased risk to animal welfare (1). However, most of the literature on human-animal relations during crisis situations has focused on natural disasters rather than disease outbreaks (2). The COVID-19 pandemic has created a situation globally where companion animals may be at increased risk of relinquishment and abandonment due to multiple interrelated factors. For example, many citizens have experienced an abrupt loss of income (3) or have begun to work at home where pets may interrupt the working day (4). Similarly, frontline workers face increased workloads and time pressures, leaving less time available to care for pets (5, 6). In times of stress and financial difficulty such as this, animal charities see increased pressure on their veterinary services and an increase in cases of cruelty, neglect and abandonment (7). At the same time, in line with government guidance, animal charities are restricting or suspending adoptions and new animal admissions and veterinarians have been forced to provide restricted services (8–11). Companion animal abandonments and relinquishments may be further increased by fears that COVID-19 can be passed on from companion animals to humans (12).

Given this unique set of circumstances, it is important to establish the impact of the COVID-19 on companion animals by establishing the prevalence of adoption and relinquishment during the pandemic and identifying risk factors for relinquishment. Furthermore, reasons for relinquishment should be assessed as they may vary from those given under normal circumstances. A distinction should be made between relinquishment, abandonment, surrender and transfer, which all refer to giving up a pet, but in different circumstances. According to Sharkin and Ruff (13), relinquishment is where a pet owner voluntarily gives up their companion animal to a shelter; transfer is where pets are given to family members or friends, surrender is where owners are required to give up their pet on an involuntary basis, and abandonment is where companion animals are left without care or any intention of resuming care. For our purposes, the term relinquishment will be used to refer to all manners in which individuals give up a pet (14, 15), unless otherwise specified.

The decision to relinquish a companion animal can take weeks and months of consideration, with pet owners trying to find an alternative home themselves before approaching an animal shelter (13, 16). In addition, Dolan et al. (16) found that when approached outside animal shelters and informed about support services, 88% of people were willing to consider alternatives to relinquishment and left the shelter with their companion animals. Therefore, it may be possible to reduce the number of relinquishments and abandonments by offering owners an alternative.

The circumstances under which a pet is acquired may influence later relinquishment decisions. Currently there is a lack of research into the association between the source of pet acquisition and the ultimate outcome for these animals (17). In particular, there is a lack of research on relinquishment outcomes for animals sourced online, and online pet acquisition more generally (18). Purchasing a pet online is of concern to animal welfare scientists and animal charities alike as they can facilitate impulsive pet acquisition and illegal puppy trading (19, 20). Time of pet acquisition may also influence risk of relinquishment. In general, newly acquired pets are more likely to be relinquished. For example, Shore et al. (21) found that 47.4% of animals relinquished to Midwestern USA animal shelters were in the home for less than a year. Similarly, Shore (22) found that 54% of returned adopted pets were sent back to the shelter within 2 weeks of acquisition, and a further 32% of pets were relinquished between 2 months to a year after acquisition. Only 7% were returned over a year after being adopted. More recently, Powell et al. (10) found that of all adopted animals from one USA shelter, 9.2% were returned within 6-months of adoption. This suggests that the majority of individuals that return pets to shelters do so either in the immediate days following acquisition or after 2 months to a year, perhaps when problems have had time to manifest themselves. There has been an increased adoption of pets during the pandemic, including an increase in impulse buying (23, 24). Consequently, there are fears that an increase in relinquishment will be seen as life returns to normal (25).

Another important influence on relinquishment risk is owner sex and gender. Recent studies that surveyed companion animal owners during the COVID-19 pandemic had a largely female participant base (26, 27). For example, Packer et al. (28) assessed puppy acquisition pre- and post-pandemic, with 90–92% of responses coming from female pet owners. The

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remaining 8–10% of participants identified as "male", "other", or "prefer not to say". Similarly, Christley et al. (6) assessed the management of pet dogs during the first UK COVID-19 lockdown with 85.7% of participants identifying as female, 14.2% identifying as male, and 0.1% identifying as "other." Christley et al. (6) highlight the need to recruit a larger sample of males to reduce sampling bias in animal behavior and welfare research. While there is evidence that the male sex relinquish pets more than the female sex (16, 29), males remain under-represented in relinquishment studies, making it difficult to arrive at a definitive conclusion.

This paper is one in a series of publications part of a larger project, "CAARP" (Companion Animal Adoption and Relinquishment during the Pandemic), which seeks to understand adoption and relinquishment of cats and dogs across several countries from the perspective of pet owners, shelter staff, and from shelter records, employing a mixture of qualitative and quantitative approaches to data collection.

The aims of this study were to:

a) Establish the prevalence of self-reported relinquishment of cats and dogs during the COVID-19 pandemic, including pet retention rates over time.
b) Identify acquisition characteristics associated with relinquishment of cats and dogs during the COVID-19 pandemic.
c) Assess the effect of gender on self-reported relinquishment of cats and dogs during the COVID-19 pandemic.
d) Identify the reasons given by companion animal owners for abandonment, relinquishment and transfer of cats and dogs during the COVID-19 pandemic.

2. Methods

2.1. Study design and participants

A cross-sectional study design was used to assess the prevalence of self-reported relinquishment of cats and dogs during the COVID-19 pandemic *via* an online survey. A sub-set of participants were invited to complete two follow-up questionnaires. Participants were recruited *via* Prolific Academic©, an online recruitment site that pays participants to take part in research. Prolific Academic has been shown to provide high quality, reliable data, and has a diverse participant pool (30). A purposive sampling method was employed by utilizing Prolific Academic's© participant screening tool. Of the 4,000 study places, 2000 places were allocated to males and 2,000 places were allocated to females. It is important to note that the Prolific Academic pre-screening tool filters respondents by male and female only by using the question: 'What sex were you assigned at birth, such as on an original birth certificate?'. Participants answer this question with one of three options: male, female, or rather not say. Therefore, when using this pre-screening tool, it is only possible to balance the study according to "male" and "female." The pre-screening tool was necessary to ensure an even split within the initial survey as we aimed to avoid the issue of female response bias often seen in survey-type research on similar topics, and in survey-type research more generally. In surveys 2 and 3, which focused on those that relinquished their pet, we ensured that participants could specify their gender. Participants were also required to be current or past pet-owners. Participants from several countries were surveyed including the UK, Ireland, Italy, Spain, France, USA, Canada and Australia, with a small number of respondents from other countries. This allowed us to explore the effects of the pandemic in countries at different stages of the pandemic. All data was collected anonymously, with each participant having a unique Prolific ID that allowed participant's responses to be matched across surveys.

2.2. Procedure

After screening participants on the Prolific Academic database, the available pool of potential participants was $N = 31{,}952$. From this pool, participants meeting the screening criteria could complete the study until $n = 2{,}000$ female and $n = 2{,}000$ male participants had completed it successfully. Survey 1 was completed on the 11th of August, 2020. Participants were first directed to a participant information sheet and completed a consent form. Participants were instructed to answer the questions with their cat or dog in mind. If they had multiple cats and/or dogs, participants were instructed to answer the questions for the pet that they most recently acquired. The initial survey was comprised of 14 questions including country of residence, animal species (cat or dog), source of acquisition, current ownership status, and whether the participant had ever considered giving up their pet. Those reporting that they had considered, or already had given up a pet, were asked about their reasons for this. After removal of partial and duplicate responses, a total of 3,945 responses were available for analysis. Participants were invited to give more detail on their experiences *via* free text responses for several questions. A sample of these responses are provided throughout the results section. A second survey was distributed 1 week later and was completed between 17th to 24th of August 2020, by those that had reported that they had considered, or had already given up a cat or dog, with $n = 181$ usable responses. Seven months later, a third survey was sent to those that have considered giving up a pet to see if there had been any changes in ownership status during this time period. This was completed between 31st March and 8th April 2021 by $n = 64$ participants. Survey 2 and 3 contained four generic questions, 24 questions for those considering giving up a pet, and 32 questions for those that have already given up their cat or dog. The detailed results from survey 2 and 3 are reported elsewhere (Carroll et al.

in preparation). For the purposes of this paper, the number of participants moving from consideration of relinquishment to actual relinquishment over the 7-month period was assessed. At each data collection point, participants verified that they had completed the survey in its entirety by providing a code which was available only on completion. Participants were then paid for their participation at an average rate of £6.50 per hour across the three surveys.

2.3. Statistical analysis

Descriptive statistics were used to analyze the demographic information collected for survey 1. A 2 x 2 Pearson's chi-square test was used to explore the relationship between categorical variables "Acquired online" (binary: Yes/No) and "Species" (binary: cat/dog). A 2 x 3 Pearson's chi-square test was used to explore the relationship between "Acquired online" (binary: Yes/No) and "When acquired" (three categories: >6 months pre-COVID; <6 months pre-COVID; after COVID declared pandemic). Binary logistic regression was used to examine the Independent variables of; "Species" (binary: cat/dog), "Source of pet" (7 categories: Adopted from a shelter/rehoming organization; Purchased from a breeder; As a gift; Directly from someone that needed to find a new home for their cat or dog; Directly from someone that was seeking temporary care for their cat or dog; The cat or dog was found as a stray; Other), "Acquired online" (binary: Yes/No) and "When acquired" (three categories: >6 months pre-COVID; <6 months pre-COVID; after COVID declared pandemic), on the Dependant Variable = "Giving up pet." Due to the small number of people that reported having already given their pet up, "Giving up pet" was changed into a binary variable where "I have considered giving up my pet" was combined with "Yes, I have already given up my pet" to form two categories "I have never considered it" vs. "I have considered or have already given up my pet." Dummy variables were created for any categorical variables with more than two categories. "As a gift" was the reference category for "Source of pet" and "Acquired more than 6 months before COVID-19 was declared a pandemic" was the reference category for "When acquired." All variables were forced into the model using the function "enter." SPSS version 25 was used for all analyses.

3. Results

3.1. Survey 1

After removal of missing responses, partially incomplete responses and those missing a Prolific ID, the sample size for the screening questionnaire was $N = 3,945$. Participants from 27 countries completed the survey. A total of 58.2% of participants were from the UK ($n = 2,305$), 20.9% from the USA ($n = 828$), 7.6% from Italy ($n = 301$), 4.3% from Canada ($n = 172$), 3.9% from Spain ($n = 156$), 1.5% from Ireland ($n = 60$), 1.2% from Australia ($n = 47$), 1.1% from France ($n = 42$) and 1.3% ($n = 52$) from other countries. Pre-existing demographic information from Prolific Academic was downloaded for each participant. This included age, ethnicity, employment status and student status. Data was missing for a number of participants due to expiration of data on Prolific Academic's system. However, from the available data, the mean age of participants was 34.7(±11.6). In total, 89.8% of participants were white, 2% were black, 3.6% were Asian, 3.6% were Mixed, and 1% selected "other." A total of 56.8% were in full time employment, 8.8% were in part-time employment, 1.6% were due to start a new job in the next month, 9.7% were not in paid work (e.g., homemaker, retired or disabled), 6.7% were unemployed, and 6.2% reported "other" for employment status. A total of 22.4% of participants stated that they were students. A total of 43.1% of participants completed the survey for a cat while 56.9% completed it for a dog. The number of participants that acquired their cat or dog *via* each source can be seen in Figure 1. Overall, most pets were purchased from a breeder (33.3%) or were adopted from a shelter or rehoming organization (30.8%).

Overall, 85.8% of pet owners obtained their pets more than 6 months before COVID-19 was declared a pandemic, 9% obtained their pets within 6 months before COVID-19 was declared a pandemic and 5.2% obtained their pets after COVID-19 was declared a pandemic. Of those that acquired their pet after COVID-19 was declared a pandemic, 42.7% were not planning on getting this pet beforehand. At the time of survey 1 (11th August 2020), 95.2% of participants had never considered relinquishment (NCR), while 4.2 and 0.5% respectively, had either considered relinquishment ($n = 168$, CR) or had relinquished their pet ($n = 21$, R). A total of 4.0% of cat owners had considered giving up their pet, while 0.5% had already given up their pet. Similarly, 4.4% of dog owners had considered giving up their pet, while 0.5% had already given up their pet.

Of the NCR group ($n = 3,774$), 87.1% acquired their pet more than 6 months before COVID-19 was declared a pandemic, 8.1% within 6 months before COVID-19 was declared a pandemic and 4.8% obtained their pets after COVID-19 was declared a pandemic.

Of the CR group ($n = 168$), 62.5% acquired their pet more than 6 months before COVID-19 was declared a pandemic, 26.2% within 6 months before COVID-19 was declared a pandemic and 11.3% obtained their pets after COVID-19 was declared a pandemic.

Of the R group ($n = 21$), 52.4% acquired their pet more than 6 months before COVID-19 was declared a pandemic, 28.6% within 6 months before COVID-19 was declared a pandemic and 19.0% obtained their pets after COVID-19 was declared a pandemic. Source of companion animal by current ownership status can be found in Table 1. A third of pets (33.7%) were

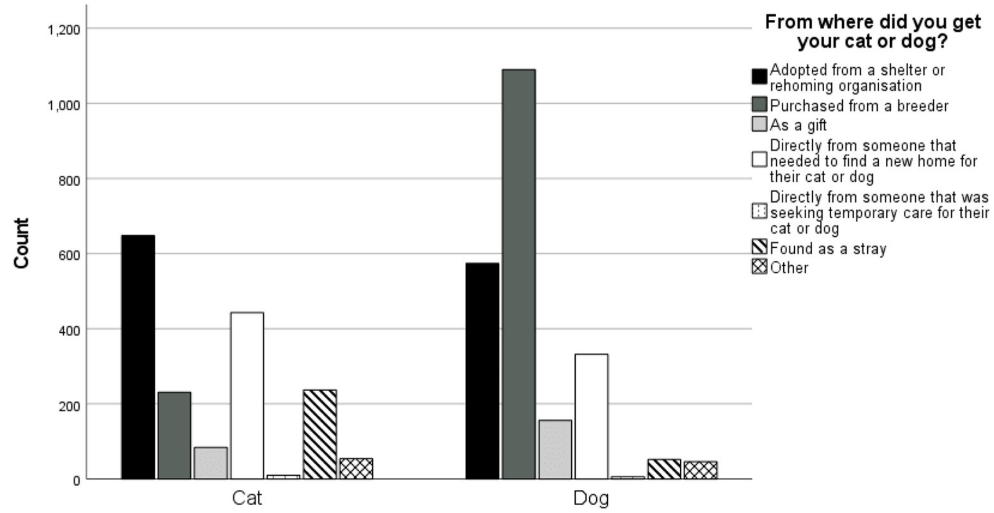

FIGURE 1
The number of participants acquiring their cats and dogs from each source.

initially found through an online source. In total, 26.6% of cats and 39.0% of dogs were originally sourced online. A 2 (Species: Cat/Dog) X 2 (Online: Yes/No) chi-square analysis revealed this difference to be significant [$X^2 = 67.026$ (1), $P < 0.001$].

The percentage of pet owners reporting that they acquired their pet online varied according to when their pet was acquired; in total, 31.7% of those that acquired their pet more than 6 months before COVID-19 was declared a pandemic sourced their pet online, compared to 43.9 and 49% of those that acquired their pet within 6 months before, and after COVID-19 was declared a pandemic, respectively. A 2 (Online: Yes/No) X 3 (time acquired: >6 months pre-COVID, <6 months pre-COVID, after COVID declared pandemic) chi-square analysis revealed this difference to be significant [$X^2 = 44.710$ (2), $P < 0.001$]. Bonferroni adjustment was used on all pairwise comparisons (6 groups) using the method outlined by Beasley and Schumacker (52). An adjusted significance threshold of $P = 0.0083$ was set. All values had a $P < 0.0083$, indicating that they were highly statistically significant.

3.2. Acquisition characteristics

For the binary logistic regression, the Independent variables: "Species" (binary), "Source of pet" (7 categories), "Acquired online" (binary), and "When acquired" (3 categories) and Dependant Variable: "Giving up pet" were entered into the model. The Hosmer and Lemeshow *P*-value ($P = 0.704$) indicated that the model is a good fit. The logistic regression model was statistically significant, X^2 (10) = 123.350, $p < 0.001$. Nagelkerke R square value of 0.096 indicated that 9.6% of the variation in the DV is accounted for by the model. There was no effect of species (cat vs. dog) on relinquishment ($P = 0.835$). Compared to pets given as a gift, there was a 38.7% decreased likelihood of relinquishment in pets sourced from shelter ($P < 0.001$), 31.2% decrease in those sourced from a breeder, and a 24.4% decrease in those acquired directly from someone that needed to find a new home for their cat or dog. There was no difference in likelihood of considering or given up a pet in those acquired directly from someone that was seeking temporary care for their cat or dog. Compared to owners who acquired their pet >6 months before COVID-19 was declared a pandemic, those acquired <6 months before COVID-19 was declared a pandemic were three times more likely to be considered for, or be, given up ($P > 0.001$). Those that were acquired after COVID-19 was declared a pandemic were two times more likely to be considered for, or be, given up ($P < 0.001$). There was a trend for greater likelihood of considering giving up or giving a pet up in those purchased online ($P = 0.074$).

3.3. Reasons for relinquishment and methods used to relinquish a cat or dog

Of those that had already given up their pet, three relinquished to a shelter (14.3%), 14 gave their pet to a new owner (66.7%) and four obtained temporary care from someone else (19.0%). Participants had the option of selecting "The cat or dog was let loose." However, no participants selected this option as a form of relinquishment. Participants were asked for the reason for considering giving up, or giving up, their cat or dog and could select as many answers as desired from 11 options. The most commonly cited reasons for considering giving up, or giving up, their cat or dog are presented in Table 2.

TABLE 1 Companion animal source by current ownership status[a].

Source of pet	Never considered relinquishment (NCR) % ($n = 3{,}756$)	Considered relinquishment (CR) % ($n = 168$)	Have relinquished (R) % ($n = 21$)
Adopted from a shelter/rehoming organization	31.2	24.4	19.0
Purchased from a breeder	33.2	35.1	38.1
As a gift	5.5	16.1	19.0
Directly from someone that needed to find a new home for their cat or dog	19.8	15.5	9.5
Directly from someone that was seeking temporary care for their cat or dog	0.3	1.5	14.3
Found as a stray	7.3	7.7	0.0
Other	2.6	0.0	0.0

[a]We did not receive any free-text responses from those that acquired their pet from someone looking for temporary care. We are therefore unsure of the reason for giving up a pet in this case but it is likely due to only temporary care being needed.

Reasons for considering giving up or giving up a pet are broken down by species (cat or dog) and relinquishment status (CR vs. R) in Table 3. Participants were invited to comment further on their reasons if they wished to do so. The most common reasons cited for considering, or given up a pet, were financial constraints. Some participants related their financial problems to the COVID-19 pandemic (e.g., *"Expenses are beyond our budget and I was made unemployed during the lockdown,"* and *"My partner was furloughed since mid March and now is at high risk of being made redundant due to the furlough scheme ending in October"*) while others referred to financial issues more generally that may or may not have been related to the COVID-19 pandemic (e.g., *"Well I would like to give away the second cat I have due to financial reasons," "Her health issues are expensive"* and *"Concerns about the financial situation"*). Health concerns specific to COVID-19 were also frequently selected as a reason for considering or having already relinquished a pet, being the second most frequently cited reason (e.g., *"I was scared that the Dog might contract COVID-19 especially when I heard that animals have likelihood of getting the disease"* and *"the fear of inadequate information on the transmission of COVID 19 is an issue for me"*). Behavioral concerns were the third most commonly cited reason for considering or having already relinquished a pet (e.g., *"My new puppy has developed into a car chaser. This is proving a huge problem and despite intensive training from a professional, it is getting worse"* and *"He's a handful"*).

3.4. Follow-up surveys

3.4.1. Further relinquishment

Those that reported having considered giving up or having already giving up their pet were invited to take part in a second survey 1 week later ($n = 189$). Data was collected between 17th and 24th of August, 2020. Of those invited, $n = 153$ usable responses were received. Five participants gave up their pet in the week between survey 1 and survey 2, moving from considering giving up their pet (CR) to actual relinquishment (R). Seven months later, participants that had competed the survey 2 were asked if they would like to participant in a revisit. Data was collected between 31st March and 8th April 2021. Of those invited, responses were received from a total of $n = 64$ participants, with no response from the remaining 104 individuals that were considering relinquishment (CR) when completing survey 2. Of these, two pets passed away, one was given to a shelter or rehoming organization, and two were given away directly to a new owner. Therefore, three participants gave up their pet over the 7 months between survey 2 and survey 3.

3.4.2. Gender

In total, 65.8% of those considering or having already relinquished their pets were male, 33.5% were female, 0.6% preferred not to say, and 0% identified as non-binary. When broken down, 65.0% of those considering relinquishment were male, increasing to 72.2% of those that have already given up their pets.

4. Discussion

4.1. Relinquishment of cats and dogs during the COVID-19 pandemic

In this study, we explored adoption and relinquishment of companion animals during the COVID-19 pandemic. Overall, considering data from the three surveys, 4.06% of participants considered giving up their pet (168/3945), 0.74% (28/3945) gave

TABLE 2 The reasons cited for considering, or actually given up a pet, in order of overall frequency.

Reason for considering or actually giving up a pet*	Overall % ($n = 189$)
1) Financial constraints have made it difficult to care for my cat or dog	44.2
2) Health concerns specific to COVID-19 (e.g., fear of cat or dog transmitting the virus to yourself or family members)	32.6
3) Behavioral concerns (e.g., house soiling, barking)	30.9
4) Safety concerns (e.g., the animal is aggressive to myself or others)	21.5
5) I feel as though I have not had enough time to properly care for the cat or dog	17.7
6) Personal reasons (e.g., divorce, my partner does not like the animal)	16.6
7) Health concerns (e.g., allergies)	16.6
8) I have increased work hours due to being an essential worker during the COVID-19 pandemic	16.6
9) The cat or dog does not get along with other pets in the household	9.4
10) I have moved house and could not bring my cat or dog	8.3
11) Other	7.2

*Participants could select as many reasons as desired.

TABLE 3 The reasons cited for giving up a pet by species (cat or dog) and consideration of relinquishment compared to actual relinquishment.

Reason for considering or actually giving up a pet*	Cat % ($n = 78$)	Dog % ($n = 101$)	Considered relinquishment (CR) % ($n = 168$)	Have relinquished (R) % ($n = 21$)
Financial constraints have made it difficult to care for my cat or dog	44.7	43.8	45.0	38.1
Health concerns specific to COVID-19 (e.g., fear of cat or dog transmitting the virus to yourself or family members)	19.7	41.9	34.4	19.0
Behavioral concerns (e.g., house soiling, barking)	31.6	30.5	30.0	38.1
Safety concerns (e.g., the animal is aggressive to myself or others)	19.7	22.9	20.0	33.3
I feel as though I have not had enough time to properly care for the cat or dog	18.4	17.1	17.5	19.0
Personal reasons (e.g., divorce, my partner does not like the animal)	21.1	13.3	14.4	33.3
Health concerns (e.g., allergies)	17.1	16.2	15	28.6
I have increased work hours due to being an essential worker during the COVID-19 pandemic	17.1	16.2	11.3	23.8
The cat or dog does not get along with other pets in the household	7.9	10.5	8.1	19.0
I have moved house and could not bring my cat or dog	6.6	9.5	7.5	14.3
Other	13.2	2.9	7.5	4.8

*Participants could select as many reasons as desired.

up their pet, and 0.2% considered and then did give up their pet (8/3945). This suggests that most pet owners that participated in this research have not considered relinquishing their pets during the time period examined. While five participants reported having given up their pet in the week between survey 1 and survey 2, only three more reported moving from consideration of relinquishment to actual relinquishment in between survey 2 and survey 3. However, only a sub-sample of participants responded to survey three. Therefore, the retention rate for all participants considering relinquishment could not be established. In any case, the relatively low percentage of CR participants that eventually relinquished pets suggests that most individuals are reluctant to give up their pets. This supports evidence from Dolan et al. (16) that a majority of pet owners are willing to consider options other than relinquishment. This suggests that there is time and opportunity to intervene in order to avoid companion animal relinquishment. However, it should be noted that most participants were white, in full time employment and largely from the UK and USA. Furthermore, those that use the Prolific Academic website to complete surveys tend to be white, have high English fluency, a medium income level, and have a third level qualification (30). Therefore, these results may not be generalizable to pet owners more generally and should be treated with caution.

While still relatively low, the number of pet owners considering relinquishment in the current study is higher than that of other studies. For example, Morgan et al. (23) reported that 2.6% of Israeli dog owners returned, or considered returning, their dogs to shelters during the COVID-19 pandemic. Brand et al. (31) examined puppy acquisition in a large sample of UK puppy owners ($N = 5{,}517$) and also reported a lower level of relinquishment at 0.9% for puppies acquired in 2019 and 1.2% for those acquired in 2020. However, these studies focused on relinquishment to shelters only. Duarte Cardoso et al. (27) assessed relinquishment of cats and dogs in Portugal that included both illegal abandonment and relinquishment to a shelter and found that 3.43% of respondents reported to have relinquished a cat or dog in the past (36/1,049), a figure more in line with our findings. In the current study, most pets that were relinquished (66.7%) were given directly to a new owner. Similarly, Hoffman et al. (32) found that, of cats and dogs acquired during the COVID-19 pandemic that were no longer with their owners, 50% of dogs and 36.8% of cats had been given to a friend, family member or neighbor. This could suggest that shelter data may under-estimate the number of individuals that give up their companion animals. In the current study, no participants selected "The cat or dog was let loose" as a means of giving up their pet. However, this may be due to social desirability bias which can occur in research that depends on owners self-reporting their experiences (33, 34). Further research is needed to identify risk factors for surrender of pets *via* a variety of means. It would also be of interest to identify the ultimate fate of animals relinquished to shelters compared to friends, family members and other third parties. However, a greater sample of relinquishers is needed.

4.2. Companion animal source

There was no effect of species (cat or dog) on risk of relinquishment. There were however, differences between cats and dogs in terms of reasons for relinquishment. These are discussed below in Section 4.5. Where the animal was sourced was related to relinquishment. Individuals that had already given up their pet were more likely to have been given the animal as a gift or directly from someone that was seeking temporary care for their cat or dog, compared to pet owners that had never considered giving up their pet. In previous studies, the source of pet has been classified according to the pet-owners' intentions on acquisition. For example, Zito et al. (35) classified acquiring a cat as a gift, or being left with the surrender by another person, as passive methods of pet acquisition. Those acquired from animal shelters or breeders were considered to have been actively acquired. Similar to Zito et al. (35), Holland et al. (36) classified those given as a gift, or dogs in need of a home, as unplanned, while those acquired in a deliberate search for a pet were classified as planned. While the proportion of participants reporting to be considering giving up or having already given up their pet in the current study was small, the intention to acquire a pet, or lack thereof, may help to explain the current study findings. Indeed, compared to those acquired as a gift, those acquired through "active" or "planned" routes (sourced from a shelter, breeder or private transaction) were at decreased risk of relinquishment. Interestingly, animals found as a stray were not at increased risk of relinquishment, despite the unplanned or passive manner of this type of companion animal acquisition. It is possible that pet owners may be less invested or attached to pets that they may have felt obliged to provide care for. Indeed, Holland et al. (36) interviewed dog owners about their motivation for pet acquisition and found that unplanned acquisitions were often from family or friends, with some pet owners actively volunteering to help, willing to help when asked, or feeling that they had little choice in the matter. Perception of choice in unexpected pet acquisition is likely important in determining future relinquishment of such pets. The increased risk of relinquishment for pets obtained as a gift is in line often voiced concern from animal charities and organizations that animals given as gifts may end up in shelters (37–39). However, this finding contrasts with most previous research, where pets given as a gift were not found to be at increased risk of relinquishment (40). For example, Scarlett et al. (41) found that only 0.3% of dog owners and 0.4% of cat owners cited "unwanted gift" as a reason for relinquishment across 12 USA animal shelters. Similarly, New et al. (29) found that very few animals (2.9%) relinquished across 12 USA animal shelters were originally given to the owner as a gift. However, Scarlett et al. (41) and New et al. (29) appear to have used the same dataset of 12 USA shelters in their studies. Furthermore, these studies were conducted approximately 20 years ago and may not be an accurate reflection of pet acquisition and relinquishment today. More recently, Weiss et al. (42) assessed relinquishment of, and attachment to, pets given as gifts. Weiss et al. (42) made a distinction between different levels of involvement in the gifting process from the pet being a surprise to the owner being involved in selecting the gifted pet, and found that gifted animals, regardless of owner involvement in the process, were not at increased risk of relinquishment. Furthermore, owners of gifted pets were just as attached to their animals as other pet owners were. A distinction between surprise gifts vs. those that were expected was not made in the current study. Montoya et al. (17) assessed the effect of the source of pet acquisition on later euthanasia risk in one Australian animal shelter and found that both adult cats and dogs were at increased risk of euthanasia if they were originally acquired as gifts. However, a "gift" included any animal acquired at no cost from family and friends. Therefore, it is impossible to determine how many of these animals were truly given as gifts and how many were actively sought out by the pet owner. Given the current study findings, more research is needed to assess the risk of relinquishment in pets acquired as gifts, with a distinction being made between unexpected gifts and those where the pet owner was actively involved in the acquisition process.

4.2.1. Online sourcing of companion animals

Overall, one third of pets in the current study were acquired *via* online means, with dogs being acquired online significantly more often than cats. There was an increase in the number of pets being purchased online over time; a third of pets acquired more than 6 months before COVID-19 was declared a pandemic were acquired online, increasing to almost 50% in those acquired post-pandemic. During the COVID-19 pandemic, Google searches for puppies increased in several countries (19) and it appears that this increase in searches has indeed been converted into increased pet acquisition. Increases in online acquisition post-pandemic may be due to shelter closures and related lockdown restrictions. However, the increase in online acquisition in the months preceding this suggests an overall increase in online sourcing of pets more generally. Similar to the current study, Packer et al. (28) found that 34.5% of 2019 puppies and 45% of 2020 puppies were acquired *via* selling websites. In terms of risk of relinquishment, there was no effect of online acquisition on relinquishment behavior ($P = 0.074$) in the current study. However, given the small sample size of those reporting to have already given up a pet, further research is needed as numerically, there was a higher prevalence of consideration of relinquishment of pets acquired online (5.8%) compared to other means (4.2%). While the increased acquisition of pets *via* online means is concerning, the internet also allows opportunity for intervention, such as regulation and monitoring (20) which should be explored in future research.

4.3. Time of acquisition

Time of companion animal acquisition was related to relinquishment. In a similar study. Hoffman et al. (32) classified pet acquisition slightly differently but uncovered comparable findings; compared to USA pet owners that did not acquire a new pet during the pandemic, those that got new pets post-pandemic were between 3 and 5 times more likely to consider relinquishment, and between 3 and 7 times more likely to actually relinquish a pet during the pandemic. However, Hoffman et al. (32) did not assess whether the relinquished pets were acquired during the pandemic or not. In the current study, it was animals that were acquired within 6 months before COVID-19 was declared a pandemic that were at greatest risk of relinquishment. Together, these results suggest that long-term pet owners were are less likely to relinquish or consider relinquishing pets during the pandemic. The reduced risk of relinquishment in those acquiring their pet post-pandemic compared to <6 months pre-pandemic may be because individuals that decided to get a pet at this time have considered the impact of lockdowns and related lifestyle changes when making their decision to acquire a pet. For example, Packer et al. (28) found that, compared to 2019, puppies were acquired more often during 2020 that suited the owner's lifestyle, were good with children, were easy to train and encouraged exercise. These decisions may have been related to the increased number of people working from home, home schooling, and restrictions on leaving the home for non-essential reasons (32, 43–45). The COVID-19 pandemic also afforded new pet owners more time to train new pets (11, 32). In contrast, owners of relatively new pets that were acquired before the COVID-19 pandemic were thrust into a new pet-ownership relationship unexpectedly, with a pet that may not have characteristics suited to the new post-pandemic environment. For example, existing pet owners may need to make adjustments to how they look after their pets such as re-training cats or dogs to toilet indoors (3). Overall, our findings suggest that it is owners of new pets that require the most support in terms of avoiding relinquishment. Those that acquired a new companion animal prior to the COVID-19 pandemic may particularly need support in helping them to adjust to the ever-evolving situation.

4.4. Pet-owner gender

A strength of the current study is the balance of male and female participants that were recruited. Despite male/female imbalances in previous studies, differences in relinquishment rates between males and females have been uncovered. For example, New et al. (29) examined the demographics of pet owners who relinquished their pets at 12 USA shelters and compared them to the demographics of a sample of USA households that contained pets. New et al. (29) found that 50.5% of dogs were relinquished by males, while 24.9% of current dog owners were male, and 38.9% of cat relinquishers were male, while 20.4% of cat owners were male. This suggests that males were more likely to relinquish animals to a shelter relative to the number that own cats and dogs. More recently, Dolan et al. (16) used a similar approach, were companion animal-relinquisher demographics were contrasted with a comparison group of pet-owners who were at the shelter to avail of low-cost/free neutering service. Dolan et al. (16) found that 38.5% of relinquishers were male, compared to 20.8% of participants in the comparison group. However, it is important to note that both of these studies grouped participants based on owner sex rather than gender. In line with our work and use of gender, Hoffman et al. (32) analyzed data on animal acquisition and relinquishment that had an even split between self-identifying males and females (47% male) and found that USA males were more likely to have acquired a pet, considered relinquishment and actually relinquished pets during the pandemic, compared to females. Our findings strengthen the evidence that males are at increased risk of relinquishing cats and dogs; 65% of those considering giving up a pet, and 72.2% of those having already given up a pet, identified as male. Future interventions to address pet relinquishment should be designed with this difference in mind. There are also other gender imbalances that need to be addressed in future work. The current work, and much of prior work, uses predominantly male and female gendered or sexed participants, meaning results may not be generalizable to the whole population. It is important that further research is conducted with even gender splits among other genders, such as non-binary, to move toward a more accurate and representative overview of pet ownership and relinquishment.

4.5. Reasons for relinquishing a pet

Financial constraints were the most mentioned reason for both giving up a pet and considering giving up a pet. Pet owners often under-estimate the financial burden associated with pet ownership (36) and companion animals may compound existing stressors in households in financial difficulty (45). For example, Eagan et al. (40) found that 10% of dog owners cited financial reasons as the reason for relinquishment of dogs to a number of Canadian shelters between 2008 and 2019. Similarly, Applebaum et al. (4) distributed an online survey to a convenience sample of 3006 USA pet owners, and found that 7% of participants reported concerns and difficulties related to financial problems and caring for their pets during the COVID-19 pandemic. In the current study, participants could select as many reasons for relinquishment as desired from a set of eleven and this may explain the increased number of participants citing financial reasons compared to previous studies. For example, finances may be related to other relinquishment reasons rather than being the primary reason in all cases. Future research should include a ranking of reasons to elucidate the most important influences on the decision to relinquish a pet. Country of residence may influence the importance of financial constraints on companion animal relinquishment. Detailed information on relinquishment by country are discussed elsewhere (Carroll et al. in preparation).

The second most cited reason for considering or relinquishing a pet was health concerns specific to COVID-19. The example given to participants for this option was "e.g., fear of cat or dog transmitting the virus to yourself or family members." The free-text responses received support the idea that pet owners fear that their pets could contract the virus and pass it to humans. Two interesting findings in relation to this were that; (1) Dog owners cited this concern more often than cat owners, and (2) participants cited this concern as a reason for considering relinquishment more often than as a reason for actual relinquishment. The first case of potential pet to human transmission was reported in a dog in Hong Kong. This is purported to have instigated a number of pet killings to avoid spreading the virus (25). However, evidence suggests that the chance of companion animals spreading COVID-19 is low (43). Indeed, COVID-19 is more likely to be transmitted from humans to their pets than vice-versa (26, 46). While there is evidence that transmission from mink to humans has occurred, most evidence shows unidirectional transition of COVID-19 from humans to several animal species including cats and dogs (47). However, this does not appear to have quelled fear in members of the public. Data collection for the current study began in August 2020, just 5 months after COVID-19 was declared a pandemic. Fears of pet to human transmission appears to have had a big impact in this time when fear and unfamiliarly with the virus was prevalent. Interestingly, it was cats that were identified by researchers as potential hosts of the virus rather than dogs (48, 49). Despite this, many more dog owners cited health concerns specific to COVID-19 as a reason for considering or relinquishing a pet. Cats can be kept indoors more easily than dogs who require exercise outside of the home (2). This may explain the difference in concerns between cat and dog owners as dogs may be difficult to keep away from members of the public who may be infected with COVID-19. More research is needed to assess differences in fear of transmission of COVID-19 between cats, dogs and their owners. Jensen et al. (50) highlight the need to distinguish between real and perceived problems. Given the lack of evidence of pet to human transmission of the virus, the risk of pet to human transition of COVID-19 is an example of a perceived problem and highlights the importance of pet owner perceptions in the relinquishment process. The public should be better educated about the true risk of transmission of COVID-19 to and from companion animals to avoid unnecessary companion animal relinquishment.

The third most cited reason for relinquishment was behavioral concerns. The importance of behavioral problems on relinquishment varies across studies and species. For example, Jensen et al. (50) assessed longitudinal data from one Danish shelter and found that behavioral problems were the most cited reason for relinquishment of dogs but were less important in cat relinquishment. In a systematic review of reasons for surrendering dogs to shelters, Lambert et al. (14) found that behavioral problems were cited as a reason in 10.8–34.2% of studies from around the globe. In the current study, behavioral problems were reported at similar levels for both species under investigation. Behavioral problems may become more apparent during lockdowns as owners have more time to notice issues. Additionally, the nature of lockdowns themselves, i.e., working from home, home schooling and limited time permitted outdoors, can negatively affect animal behavior, both at the time and into the future as restrictions ease. For example, more time spent at home may increase the risk of aggression toward children (51). Indeed, Tulloch et al. (44) found that the incidence of dog bites increased at pediatric emergency departments during UK lockdown periods. Furthermore, Brand et al. (31) found that puppies purchased during the pandemic were left alone less often and had less experience of public spaces, and humans and dogs outside of the family unit. Poor socialization can, in turn, increase the risk of aggression in dogs in the future (31). What's more, problems may be exacerbated by inexperience with pet ownership in those that acquired a pet post-pandemic (43). The current study findings suggest that behavioral concerns remain as a key consideration in companion animal relinquishment. Jensen et al. (50) highlight the importance of distinguishing between owner-related and pet-related reasons for relinquishment as they may require varying solutions. In the current study, the main reasons for considering relinquishment, or actually relinquishing a pet, were for financial, health concerns related to COVID-19, and behavioral problems for dogs. This suggests that interventions that target both animal behavior and human expectations are required.

5. Conclusion

The concern around relinquishment of companion animals in recent times has focused on those acquired during the pandemic. Our findings suggest that cats and dogs acquired post-pandemic are indeed at increased risk of relinquishment compared to those acquired over 6 months beforehand. However, it was those acquired in the 6 months before the COVID-19 pandemic that were at particular risk of relinquishment. Greater support is needed for this group of pet owners. This study provides strong evidence that males are at increased risk of relinquishing pets compared to females. The reasons for this difference should be explored further and relinquishment interventions should be designed with this gender difference in mind. Most individuals that relinquished a pet, gave the animal away to a new owner. More research is needed into this type of relinquishment to establish the true prevalence of companion animal relinquishment, and to establish the ultimate outcome of pets relinquished in this manner. Fear of pet to human transmission of COVID-19 is playing a role in companion animal relinquishment, despite the lack of evidence of pet to human transmission of the virus. Greater education of members of the public on this topic is required. There appears to be a shift toward online acquisition of pets in recent times. More research needed on effect of online acquisition on relinquishment of cats and dogs. Contrary to previous research, pets acquired as gifts were found to be at increased risk of relinquishment. More research on this is needed, given the conflicting findings within the literature. Finally, less than 10% of the variation in relinquishment intention was explained by the factors assessed in the current study. This suggests that other factors are also at play. Owner demographics, such as home ownership status and family composition, will be explored in a separate publication as part of the CAARP project.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Queen's University Belfast Faculty Research Ethics Committee (EPS 20_111). The patients/participants provided their written informed consent to participate in this study.

Author contributions

GC and CR contributed to conception and design of the study and acquired the funding for this research. GC and AT performed the data analyses. GC wrote the first draft of the manuscript and provided supervision. AT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data evaluation of broiler chicken rearing and slaughter—An exploratory study

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To process and evaluate the data from broiler fattening and slaughtering, we investigated the production data of 107 straight run flocks of the commercial meat-type breed Ross 308 (Aviagen, EU). All flocks were raised and slaughtered in Germany and the average slaughter age was 37 days. The health outcomes of interest were mortality, average weight, and the slaughter results. First-week mortality, cumulative mortality, stocking density, flock size, season, production week of the parental flock, farm, antibiotic treatment, and the interaction between antibiotic treatment and season were considered as possible influencing factors. The average first-week mortality (FWM) and cumulative mortality percentages were 0.66 and 2.74%, respectively. First-week mortality was influenced by flock size, production week of the parental flock, and the interaction between antibiotic treatment and season, whereas cumulative mortality was influenced by antibiotic treatment, farm, and first-week mortality. The average weight (mean 2.30 kg) was influenced by season, stocking density, flock size, farm, and the interaction between antibiotic treatment and season. The condemnation rate was on average 1.48%, with the most common causes being deep dermatitis (mean 0.63%), ascites (mean 0.53%), and not suitable for production/general disease (mean 0.25%). Several factors influenced the causes of condemnation, with season being the most predominant one, followed by the interaction between antibiotic treatment and season, the antibiotic treatment alone, and stocking density.

KEYWORDS

poultry, mortality, condemnation, slaughter, rejection

Introduction

Although vegetarian nutrition is becoming more popular these days, broiler meat is still favored because it is low in fat, easy to prepare, and affordable. The German meat industry produced 623,165,170 broilers in 2020, corresponding to a weight of 1,066,528,075 tons (1). Because broiler meat is an essential part of the meat produced in Europe and worldwide, the enormous effort put into research on broiler welfare is explainable and reasonable (2).

For poultry production, mortality records are of major importance because they may reflect possible disease incidences (3). Furthermore, performance and health data of broiler flocks must be collected regularly during the fattening period (4). In Germany, mortality must be routinely recorded in broiler flocks according to Article 19 of the German Order on the Protection of Animals and the Keeping of Production Animals (5). This regulation requires the farmer to document the daily mortality rate and calculate the cumulative mortality rate (5). The first-week mortality (FWM) in the life of broilers is a significant factor, which is widely assessed in poultry production (6) and can serve as an indicator of the health status and performance of the broiler flock during the fattening period (7). Therefore, there is a need to put more effort into the first 7 days of the chicks' life to make sure they develop good drinking and feeding behavior and thus quickly maximize their opportunity for growth. However, there are changes in the weekly mortality percentages throughout the fattening process (7). Because large numbers of broilers are processed, even incidents of small effects during the fattening period can be of large economic importance to the meat industry (8). Thus, high mortality in broiler flocks is associated with a lower income for the broiler farmers (7) and a considerable financial loss caused by mortality and injuries from the enormous number of broilers that are slaughtered, which represents a lack in animal welfare that must be addressed (9).

At the time of slaughter, the weight of the animals is recorded and can therefore be regarded as a standardized and objective measure of the health of a broiler flock; furthermore, the weight data can reveal poor flock uniformity (10). At the processing plant, the carcasses and associated by-products undergo a final post-mortem inspection. All external surfaces, body cavities, and by-products are examined and the findings recorded (11). Those recordings can be used for surveillance purposes. The feedback the farmer receives from slaughterhouses for each flock includes information on flock performance, condemnation rate, and causes of condemnation. By use of these data, improvements in the production chain can be made, not only by the farmer to enhance the production but also by the veterinarians for advising and consulting or by the personnel and veterinarians working at the processing plant, who perform a risk-based meat inspection. In addition, this information allows scientists to improve their fields of study (12). Several authors have already researched possible factors that may influence the results of broiler fattening. Van Limbergen et al. (13) showed in their study the influence of many factors of broiler farm management and housing on broiler health and performance, as well as the impact of health problems caused by septicemia, coccidiosis, and dysbacteriosis. De Jong and van Riel (4) found that causes of condemnation at slaughter, uniformity of carcass weight, first-week mortality, and cumulative mortality all showed seasonal variations, with the best performances obtained when the broilers were farmed during the summer months.

Mean values of 1.36% (12) and 1.10% (14) were reported as condemnation percentages in broilers, and Salines et al. (12) mentioned generalized constipation, cachexia, and non-purulent skin lesions as the main findings in meat inspection. In addition, Alfifi et al. (14) found scratching and dermatitis as the most common reasons for condemnation, followed by ascites.

The objective of this study was to combine data on broiler chicken rearing and slaughtering from the same flocks and to identify factors that may influence the mortality during the fattening period, the slaughter weight, and the causes of condemnation recorded at the processing plant. This enabled the identification of farm management factors to inform poultry production best practices. This study provides an overview of current broiler rearing and slaughter data in an average German broiler production system.

Materials and methods

Study population and design

For this study, flocks from two farms in Germany were analyzed: five barns from farm 1 and six barns from farm 2 were included in the study. Some flocks had to be excluded from the study because of missing data. Both farms belonged to the same company, were in the same area, only 18 km apart from each other, and were led by the same operation manager.

Barns

All barns had a small room serving as a sluice and were entered with farm-specific clothes and barn-specific shoes. All barns were emptied, cleaned, and disinfected in the service period between the fattening periods. The authorized broiler numbers for the barns were 10,100 (barn 1, farm 1), 9,700 (barn 2, farm 1), 10,100 (barn 3 and 4, farm 1), 29,200 (barn 5, farm 1), 41,500 (barn 1–4 and 6 of farm 2), 19,300 (barn 5, farm 2) with an average 27,000 birds per flock. All barns were equipped with round feeders and feed was supplied *ad libitum*. The feeder space differed according to the number of birds housed, resulting in feeder space per bird according to legal regulation. There was a difference in the litter material used on the two farms; farm 1 used straw granulate for bedding, and farm 2 used straw pellets.

To keep the temperature in the desired range, all barns were equipped with spray cooling systems and a gas heating system. Before housing, the barns were heated up to an air temperature of 33°C. All barns were closed barns with a forced ventilation system. The ventilation capacity ranged between 6.7 to 14.5 m^3 per housed animal and hour and was adapted to the size of the birds and their necessity by a temperature and humidity control system.

Both farms received their chicks from the same hatchery and the proximity from hatchery to farm was similar (287 km for farm 1 and 276 km for farm 2). Both farms used the same vaccination program: vaccination program against Infectious Bronchitis Virus (IBV) where a primer (half dose) and booster (full doses) were applied at the hatchery and at day 10 on the farm, Newcastle Disease Virus (NDV) and Infectious Bursal

Disease (IBD, also known as Gumboro) were applied *via* drinking water at day 16 on the farm.

Data generation

The first chicks were housed in January 2019 and the last in June 2020, and 107 fattening periods were considered in the study. The following number of fattening periods were included for each barn: barn 1, farm 1: 8; barn 2 farm 1: 8, barn 3, farm 1: 8; barn 4 farm 1: 8, barn 5, farm 1: 9; barn 1 farm 2: 11, barn 2, farm 2: 11; barn 3 farm 2: 11, barn 4 farm 2: 11; barn 5 farm 2: 11; barn 6 farm 2: 11. The mean stocking density was 33.86 kg/m^2, with a minimum of 30.08 and a maximum of 39.43 kg/m^2. For further analysis, the flock size was categorized into small ($\leq$11,000 broilers), medium ($>$11,000 to $\leq$30,000 broilers), and large ($>$30,000 to $\leq$42,000 broilers). Regarding the genotype, only Ross 308 (Aviagen, EU) broilers were included in this study. All flocks were of straight run. Only birds from the final depopulation were included in the study, and the average duration of the fattening period was 37 days (minimum 35 days and maximum 40 days).

The catching time was evaluated and was divided into day ($>$4 am until $<$9 pm) and night ($\geq$9 pm until $\leq$4 am). In total, 82 flocks were caught at night time and 25 flocks were caught during the day. All birds were brought to the same processing plant with a transport distance from farm 1 of 229 km and 242 km from farm 2. Slaughtering took place from February 2019 to July 2020, using a controlled atmosphere stunning as the stunning method. The meat inspection was done by official authorities and the staff of the processing plant, according to Commission Implementing Regulation (EU) 2019/627 (11).

To analyze the influence of the seasons, the following seasons were used: winter (= December, January, February), spring (= March, April, May), summer (= June, July, August), and fall (= September, October, November). The production week of the parental flock was also included in the study and varied from 3 to 38 weeks.

The information and data used in this study were obtained from the following four sources: (i) farm record: date of housing, number of chicks per flock (flock size), mortality (FWM and cumulative mortality), litter material, catching time; (ii) delivery note: production week of the parental flock, genotype; (iii) delivery and application documents from the veterinarian: antibiotic treatment (name of the drug, date of application, number of days of usage, age of the broilers when the drug was given, diagnosis); (iv) slaughter records: date of slaughter, average weight, number of birds processed, condemnation rate, dead-on-arrival (DOA) rate, and causes of condemnation. The latter included deep dermatitis, ascites, not suitable for production/general disease, hepatic changes, polyserositis, underdevelopment/emaciation, other pathologic findings/hematoma/injuries, and changes in color/odor/texture. Before the start of each antibiotic treatment, a clinical examination of the flock was performed by a veterinarian. The clinical diagnoses were used, together with pathological findings of dead or culled birds, a microbiological examination and antibiogram were carried out to find the most suitable antibiotic. All microbiological examinations and antibiograms were performed at the laboratory of the veterinarian in charge and the data sets were provided by the farmer.

Statistical analysis

All information about the broilers, considering the whole fattening period and the details about the slaughtering of the flocks, was provided by the farmer and handed in on paper. The data were collected and transformed into an Excel table and then transferred to IBM SPSS Statistics software, version 27 (SPSS Inc., Chicago, IL, United States). Data inputs were validated and descriptive statistics were obtained to validate the information, followed by a calculation of the means, the minimum and maximum values, and the standard deviation. Histograms on all relevant farm-level and slaughter variables were conducted and checked visually for potential errors, extreme values, and normal distribution.

All data provided were checked for their influence on the dependent variables and independent variables with at least one significant *p*-value ($\leq$0.05) were included in the model. This led to eight independent variables plus the interaction between season and antibiotic treatment as a random effect (Figure 1). The farm was included in the model to consider any influence of the difference in the litter that was used. First-week mortality, cumulative mortality, average weight, and the causes of condemnation (condemnation rate in total, DOA rate, deep dermatitis, ascites, not suitable for production/general disease, hepatic changes, polyserositis, underdevelopment/emaciation, other pathologic findings/hematoma/injuries, and changes in color/odor/texture) were each used in a multivariable model as the dependent variable. For a more precise model, backward selection was performed with regard to the corrected R^2. The model with the highest R^2 was chosen for further interpretation. To check the influence of the catching period, an ANOVA was additionally performed and evaluated for the condemnation rate in total and the DOA rate. For the ascites findings, an additional ANOVA was performed to check the influence of the average weight.

$P \leq 0.05$ were considered significant. By using the conditional studentized residual plots, the residuals and the assumptions of homogeneity of variance were predicted and checked visually for normal distribution.

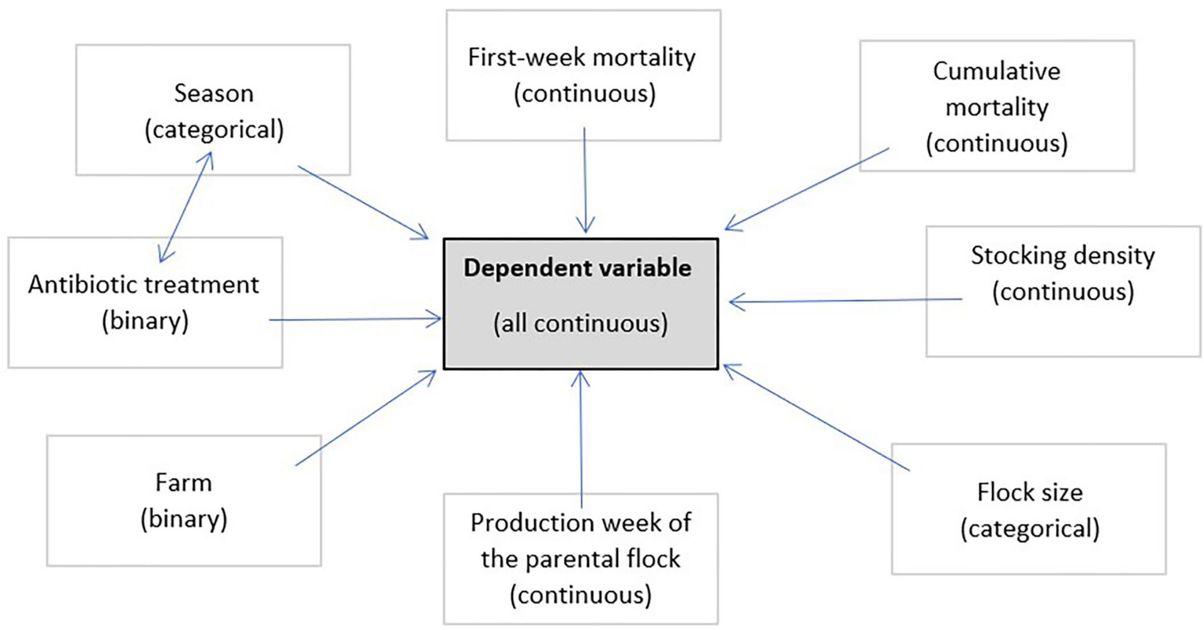

FIGURE 1
Overview of the independent variables and their characteristics used in the multivariable model. First-week mortality, cumulative mortality, average weight, and the causes of condemnation (condemnation rate in total, DOA rate, deep dermatitis, ascites, not suitable for production/general disease, hepatic changes, polyserositis, underdevelopment/emaciation, other pathologic findings/hematoma/injuries, and changes in color/odor/texture) were each used as the dependent variable.

TABLE 1 Mean values and standard deviation of first-week mortality, cumulative mortality, and average weight, separately listed according to season and use of antibiotic treatment.

	n	First-week mortality (%)	Cumulative mortality (%)	Average weight (kg)
Season				
Fall	16	0.46 ± 0.11	2.20 ± 0.67	2.38 ± 0.09
Winter	23	0.69 ± 0.27	2.69 ± 0.65	2.31 ± 0.12
Spring	41	0.72 ± 0.30	3.15 ± 1.79	2.27 ± 0.10
Summer	27	0.66 ± 0.24	2.47 ± 1.21	2.30 ± 0.17
Antibiotic treatment				
No	77	0.67 ± 0.25	2.52 ± 1.32	2.33 ± 0.12
Yes	30	0.64 ± 0.30	3.28 ± 1.31	2.24 ± 0.12
Total	107	0.66 ± 0.28	2.74 ± 1.36	2.30 ± 0.13

n, number of examined flocks.

Results

Mortality

The descriptive results for FWM, cumulative mortality, and average weight are presented in Table 1. The overall mean first-week mortality was 0.66%. As shown in Table 2, the FWM was influenced by flock size ($p < 0.001$), production week of the parental flock ($p = 0.044$), and the interaction between antibiotic treatment and season ($p = 0.004$). Smaller flock sizes and parental flocks in an earlier production week led to a lower FWM. In addition, the FWM was higher in flocks without reported antibiotic treatment (0.67%) than in flocks reported to have been treated with antibiotics (0.64%).

The overall mean cumulative mortality was 2.74%. Considering the seasons, the highest rate was observed in spring (3.15%) and the lowest in fall (2.20%), but seasonal variations observed did not differ and were thus excluded from the final model. Flocks that had been treated with antibiotics had higher cumulative mortality (3.28%) than flocks that had not been treated (2.52%) ($p = 0.026$; Table 2). The statistical model showed an influence of FWM ($p = 0.003$), farm ($p = 0.008$),

TABLE 2 *P*-values of the multivariate analysis using a generalized linear model for the dependent variables first-week mortality, cumulative mortality, and average weight.

Independent variable	Dependent variable		
	First-week mortality	Cumulative mortality	Average weight
	R^2 0.284	R^2 0.185	R^2 0.648
Season	–	–	0.002
First-week mortality		0.003	–
Cumulative mortality	–		0.085
Stocking density	–	–	<0.001
Flock size	<0.001	0.054	0.002
Production week of the parental flock	0.044	–	–
Antibiotic treatment	–	0.026	–
Farm	–	0.008	0.003
Interaction Antibiotic treatment * Season	0.004	–	<0.001

TABLE 3 Causes of condemnation (%), mean, minimum, maximum, and standard deviation (SD).

Cause of condemnation	*n*	Mean	Minimum	Maximum	SD
Condemnation rate in total	107	1.48	0.12	4.63	0.89
Deep dermatitis	106	0.63	0.04	3.13	0.67
Ascites	106	0.53	0.19	0.53	0.28
Not suitable for production/general disease	107	0.25	0.00	1.66	0.23
Dead on arrival	107	0.17	0.03	0.66	0.09
Hepatic changes	106	0.11	0.00	0.62	0.09
Polyserositis	106	0.09	0.00	2.10	0.25
Underdevelopment/emaciation	106	0.05	0.00	0.41	0.07
Other pathologic findings/hematoma/injuries	106	0.05	0.00	0.30	0.04
Changes in color/odor/texture	106	0.04	0.00	0.30	0.04

n, number of examined flocks.

and antibiotic treatment ($p = 0.026$) on cumulative mortality (Table 2).

Average weight

The average weight of the broilers of all flocks was 2.30 kg (Table 1). Considering the seasons, the heaviest birds were found in fall (mean 2.38 kg) and the lightest in spring (mean 2.27 kg) ($p = 0.002$; Table 2). The average weight was higher in the flocks that had not been treated with antibiotics (mean 2.33 kg) than in those that had been treated (mean 2.24 kg), the difference was, however, not significant, only the interaction between antibiotic treatment and season was supported ($p < 0.001$). Stocking density ($p < 0.001$), farm ($p = 0.003$), and flock size ($p = 0.002$) also had an effect on the average weight (Table 2). A higher stocking density was associated with a higher average weight. Flocks categorized as large had a lower average weight than flocks categorized as small, while medium flocks had the highest average weight.

Causes of condemnation

The condemnation percentage in our study was 1.48%, with deep dermatitis (mean 0.63%) and ascites (mean 0.53%) being the major causes of condemnation (Table 3). The condemnation percentage was influenced by the season ($p < 0.001$), the production week of the parental flock ($p = 0.008$), and the use of antibiotics ($p = 0.002$; Table 4). The highest condemnation percentages were found in fall (mean 1.90%) and the lowest in winter (mean 1.34%) and spring (1.31%). The DOA percentage in our study showed a mean value of 0.17% and was influenced by FWM ($p = 0.039$), flock size ($p = 0.046$), the use of antibiotics ($p = 0.018$), and the interaction between antibiotic treatment and season ($p = 0.040$; Table 4). Flocks with a high FWM had a higher DOA percentage. Flocks without antibiotic treatment had

TABLE 4 *P*-values of the multifactorial analysis using a generalized linear model for the dependent variables condemnation rate in total, dead-on-arrival (DOA), deep dermatitis, ascites, not suitable for production/general disease, and hepatic changes.

Independent variable	Dependent variable					
	Condemnation rate in total	DOA	Deep dermatitis	Ascites	Not suitable for production/ general disease	Hepatic changes
	R^2 0.174	R^2 0.197	R^2 0.146	R^2 0.392	R^2 0.206	R^2 0.207
Season	<0.001	0.167	0.021	<0.001	0.044	0.062
First-week mortality	0.133	0.039	–	–	0.337	–
Cumulative mortality	–	0.088	–	–	0.128	0.187
Stocking density	0.162	0.139	0.133	0.226	<0.001	<0.001
Flock size	–	0.046	–	0.002	0.068	0.102
Production week of the parental flock	0.008	0.236	0.002	–	–	–
Antibiotic treatment	0.002	0.018	0.285	<0.001	0.207	0.106
Farm	–	0.198	–	–	–	–
Interaction antibiotic treatment * season	–	0.040	–	<0.001	–	–

lower DOA percentages than flocks with antibiotic treatment. An influence of the catching time was found in the additional ANOVA, showing that flocks that were caught during the night time had a lower DOA percentage compared to the flocks caught during day time ($p< 0.001$). Deep dermatitis was the most common cause of condemnation during slaughter in our study and was influenced by the season ($p = 0.021$) and the production week of the parental flock ($p = 0.002$; Table 4). The pathological finding was that ascites examined during slaughter was strongly influenced by the season ($p < 0.001$), the flock size ($p = 0.002$), antibiotic treatment ($p < 0.001$), and the interaction between antibiotic treatment and season ($p < 0.001$; Table 4). Differences between the seasons on the ascites findings could be seen, with the highest findings in winter (mean 0.71%) and fall (mean 0.61%). In spring (mean 0.45%) and summer (0.43%), fewer broiler carcasses were condemned because of ascites. The results of the ANOVA with the influence of average weight on ascites showed an influence of average weight ($p = 0.038$). The pathological findings were that not suitable for production/general disease ($p < 0.001$) and hepatic changes ($p = 0.001$) were both influenced by the stocking density, and another finding was that not suitable for production/general disease was additionally influenced by the season ($p = 0.044$). Although polyserositis was only recorded in 0.09% of the condemned carcasses (Table 3), the multivariate model revealed influences for the factors season ($p < 0.001$), antibiotic treatment ($p < 0.001$), and the interaction between antibiotic treatment and season ($p < 0.001$; Table 5). Underdevelopment/emaciation was influenced by season ($p = 0.010$) and by stocking density ($p < 0.001$), whereas other pathologic findings/hematoma/injuries were influenced by season ($p = 0.014$), cumulative mortality ($p = 0.015$), production week of the parental flock ($p < 0.001$), and the interaction between antibiotic treatment and season ($p = 0.021$; Table 5). The least common finding (changes in color/odor/texture: mean value of 0.04%; Table 3) was influenced by season ($p = 0.012$), stocking density ($p = 0.048$), flock size ($p = 0.013$), antibiotic treatment ($p = 0.022$), and the interaction between antibiotic treatment and season ($p = 0.018$; Table 5).

Antibiotic treatment

Regarding the antibiotic treatment, 30 of the 107 flocks were reported to have been treated with antibiotics during the fattening period, whereas 77 had not been treated (Table 6). Of the 30 flocks with antibiotic treatment, 25 were categorized as large flocks (>30,000 to ≤42,000 broilers), and 5 flocks were categorized as medium ones (>11,000 to ≤30,000 broilers). None of the small flocks (≤11,000 broilers) had been treated. We observed differences in the seasons, with most flocks with antibiotic treatment housed in spring ($n = 18$), whereas fewer flocks received antibiotic treatment in winter ($n = 8$), summer ($n = 3$), and fall ($n = 1$). Eight flocks were treated two times within the fattening period (one flock in fall, one flock in summer, and six flocks in winter), and two flocks were treated three times (both in spring). The other 20 flocks were treated one time with antibiotics during the fattening period.

Discussion

This study evaluated flock production and slaughter records and assessed factors influencing various production metrics including FWM, cumulative mortality, average weight, and condemnation percentages. Our analysis is suggestive that FWM

TABLE 5 *P*-values of the multifactorial analysis using a generalized linear model for the dependent variables polyserositis, underdevelopment/emaciation, other pathologic findings/hematoma/injuries and changes in color/odor/texture.

Independent variable	**Dependent variable**			
	Polyserositis	**Underdevelopment/emaciation**	**Other pathologic findings/ hematoma/injuries**	**Changes in color/ odor/texture**
	R^2 **0.278**	R^2 **0.192**	R^2 **0.331**	R^2 **0.217**
Season	<0.001	0.010	0.014	0.012
First-week mortality	–	–	–	–
Cumulative mortality	0.147	0.224	0.015	–
Stocking density	–	<0.001	0.109	0.048
Flock size	–	0.227	0.146	0.013
Production week of the parental flock	–	0.168	<0.001	–
Antibiotic treatment	<0.001	–		0.022
Farm	0.272-	–	–	–
Interaction antibiotic treatment * season	<0.001	–	0.021	0.018

(mean: 0.66%, n = 107 flock cycles) was influenced by flock size, production week of parental flock, and the interaction between antibiotic treatment and season. The interaction between antibiotic treatment and season described whether antibiotics were used in the flock during the fattening period and the differences between the number of treatments per season and its effect on the FWM. Previous studies reported higher percentages of 0.94, 1.03, 1.10, and 1.82% than we observed (6, 13, 15, 16). The relatively low FWM in the flocks of our study might suggest that the chicks were of good quality and health, and the management and brooding conditions were good. According to Yerpes et al. (6), the FWM can be used as an important production criterion in poultry production. In our study, the first-week mortality differed between flocks, and several influencing factors were identified, underlining the statement by Yerpes et al. (6). Regarding the factors which can influence first-week mortality in broilers, van Limbergen et al. (13) cited floor quality, ventilation type, presence of other professional activities of the farmer and neonatal septicemia as the most common ones. In addition, Heier et al. (17) described a relationship between stocking density and FWM, whereby flocks with higher stocking density had lower mortality in the first week after housing. In our study, the stocking density did not affect first-week mortality, but the flock size did. The highest FWM was observed in flocks categorized as medium while the lowest was found in flocks categorized as small. This could be because smaller flocks had a lower infection pressure and the broilers may also have less stress due to the smaller number of birds. Yerpes et al. (6) identified the age of the parental flock, gender of the chicks, genotype, type of broiler housing, presence of drip cup, egg storage, and study year as factors influencing the FWM. An influence of the production week of the parental flock was also observed in our study: the FWM increased if the production week of the parental flock increased, also confirming the findings of O'Dea et al. (18). These authors described a higher mortality in broiler chicks produced by 57-week-old parental flocks than in those produced by 28- and 43-week-old parental flocks. After the broilers reached the age of 3 weeks until the time of slaughter, the cumulative mortality in all flocks was the same, regardless of the age of the parental flocks (18). These results were similar to ours. In contrast, McNaugton et al. (19) found that chicks from 29-week-old parental flocks showed higher mortality than those from 58-week-old parental flocks. Yerpes et al. (6) reported an influence of the season on first-week mortality and emphasized the importance of controlling and minimizing seasonal fluctuations in the hatchery, on the broiler farms, or during the transport to reduce the influence of seasonal fluctuations on mortality. Although we observed differences in the mean percentages between the seasons, these variations were not significant. In the study by Yerpes et al. (6), first-week mortality was highest in fall and winter. In contrast, our findings suggested FWM to be lowest in the fall. However, the study by Yerpes et al. (6) took place in Spain, whereas our study took place in Germany. Thus, the weather conditions differ, and the differences between summer and winter may be of a higher extreme in Spain than in Germany, because of the influence of Spain's microenvironments (6). Petracci et al. (8) reported seasonal FWM percentages similar to our results, with the highest percentages in spring and the lowest in fall (8).

The average cumulative mortality in our study was 2.74% (n = 107 flock cycles) and was influenced by the FWM, the use of antibiotics during fattening, and the farm.

The cumulative mortality in our study was lower than the values determined in previous studies and the EFSA also states cumulative mortality of 5.00% to be usual (20). In one study, Kittelsen et al. (15) analyzed data from 59 broiler flocks

TABLE 6 Antibiotic treatments ($n = 30$): name, active ingredient, diagnosis, withdrawal time of drug (days), number of treatments, week of life when treated, season, and size of the treated flock.

Antibiotic name	Active ingredient	Diagnosis	Withdrawal time of drug (d)	Number of treat-ments	Week of life when treated	Season	Flock size
Ampiciph	Ampicillin	A	6	1	3	Winter	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	3	Winter	Medium
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	1	Spring	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	1	Spring	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	1	Spring	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	1	Spring	Medium
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5		1	Spring	Large
Belacol	Colistin sulfate	*E. coli* infection	2		4		
Octacillin	Amoxicillin	E	1		5		
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	1	Spring	Large
Phenocillin	Phenoxymethyl-penicillin	E	2	1	2	Spring	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	2	Summer	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5	1	3	Summer	Large
Fluonix	Enrofloxacin	Polyserositis	7	2	3	Summer	Large
Metaxol	Trimethoprim, sulfamethoxazole	UYSI	5		2		
Belacol	Colistin sulfate	*E. coli* infection	2	2	5	Fall	Medium
Suramox	Amoxicillin	A	1		5		
Octacillin	Amoxicillin	*E. coli* infection	1	2	5	Winter	Large
Belacol	Colistin sulfate	A	2		4		
Octacillin	Amoxicillin	*E. coli* infection	1	2	5	Winter	Large
Belacol	Colistin sulfate	A	2		4		
Octacillin	Amoxicillin	*E. coli* infection	1	2	5	Winter	Large
Belacol	Colistin sulfate	A	2		4		
Ampiciph	Ampicillin-	A	6	2	3	Winter	Large
Belacol	Colistin sulfate	*E. coli* infection	2		5		
Ampiciph	Ampicillin-	A	6	2	3	Winter	Large
Belacol	Colistin sulfate	*E. coli* infection	2		4		
Ampiciph	Ampicillin-	A	6	2	3	Winter	Large
Belacol	Colistin sulfate	*E. coli* infection	2		5		
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Octacillin	Amoxicillin	A	1	1	5	Spring	Large
Octacillin	Amoxicillin	A	1	1	3	Spring	Medium
Belacol	Colistin sulfate	*E. coli* infection	2	3	3	Spring	Large
Octacillin	Amoxicillin	A	1		4		
Octacillin	Amoxicillin	A	1		5		
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Large
Lincospectin	Lincomycin, spectinomycin	E	5	1	1	Spring	Medium

E, Enteritis; A, Arthritis; UYSI, Umbilical yolk sac inflammation; *E. coli*, Escherichia coli. The flock size was categorized as follows. Small: ≤11,000 broilers; medium: >11,000 to ≤30,000 broilers; large: >30,000 to ≤42,000 broilers.

of different farms and found a mean cumulative mortality percentage of 2.94%; as in our study, all broilers were Ross 308 (Aviagen, EU), and the mortality data were taken from farm evaluations. In another study, Kittelsen et al. (16) analyzed data from 61 straight run Ross 308 (Aviagen, EU) flocks, which they investigated at the processing plant, and found a mean cumulative mortality percentage of 3.0% (16). Jacobs et al. (21) and van Limbergen et al. (13) reported an overall mortality of 3.2 and 3.8%, respectively. These comparisons further support the importance of good husbandry practices to optimize the health of the flock. Our results give the impression of good management and health of the flocks evaluated in the presented study because both the average first-week mortality and the average cumulative mortality were lower than those in the studies reported.

It is reported that neonatal septicemia is one of the factors influencing overall mortality (13). In line with the findings of van Limbergen et al. (13), Tabler et al. (22), and Campe et al. (23), our analysis revealed that the cumulative mortality was influenced by the first-week mortality. Flocks with a high FWM showed higher cumulative mortality over the fattening period, which might indicate that health problems during the early stages of the life of the bird could have long-term consequences. Tabler et al. (22), for example, found that high mortality within the first days of the chicks' life resulted in a flock with poorer health status in general and with more animals susceptible to infections. Furthermore, they described a problem with uniformity in flocks with high early mortality. In the study by Campe et al. (23), mortality was additionally affected by the length of the fattening period, the hatchery, and an interaction between litter type and weather (23). In our study, the farm also influenced the cumulative mortality, thus indirectly indicating that farm-specific aspects, such as litter, hygiene, and management in general could have an influence. In line with the findings of Feddes et al. (24), neither the stocking density nor the flock size influenced the cumulative mortality in our flocks. To reduce the cumulative mortality in broiler flocks, Yassin et al. (7) recommended that farmers reduce the number of chicks, and Buragohain and Karlita (3) emphasized the importance of providing water and feed of good quality and practicing good management in the first days of the chicks' life.

The production week of the parental flock did not affect the cumulative mortality in the broiler flocks of our study, confirming the findings of Jacobs et al. (21) and Ulmer-Franco et al. (25). With regard to the season, the lowest cumulative mortality was observed in fall (2.20%) and the highest in spring (3.15%). The lowest and the highest FWM were also found in fall and spring, respectively. However, in contrast to the findings of Vieira et al. (26), no influence of seasonal differences was observed. The average slaughter weight of the Ross 308 broilers in our study was 2.30 kg (n = 107 flock cycles) and was influenced by stocking density, flock size, farm, season, and the interaction between antibiotic treatment and season. With an increase in the stocking density, the average weight increased as well, which is contrary to the results of Dozier et al. (27). An explanation might be, that broilers of flocks with a higher stocking density were less active due to the reduced space per bird and thus achieved a higher weight. The EU Broiler Directive 2007/43/EC requires that the maximum stocking density does not exceed 42.00 kg/m^2 at any time (28), whereas the German Order on the Protection of Animals and Keeping of Production Animals only allows a maximum stocking density of 39.00 kg/m^2 (5). In our study with the average stocking density of 33.86 kg/m^2 and a range from 30.08 up to 39.43 kg/m^2, a range of density was observed. Campe et al. (23) observed an influence of stocking density on the body weight of broilers, whereas Feddes et al. (24) found no such relationship because the mean weight of the broilers in their study did not differ between flocks of low and high stocking density. However, the flocks with the lowest stocking density in their study were less uniform than those with higher stocking densities (24). Regarding the influence of the flock size, the highest weight was found in medium flocks and the lowest weight was found in large flocks. This could be explained by the fact, that birds in larger flocks had more stress to cope with and less access to resources as already reported by Dozier et al. (27), and stress, in particular, can be the cause of reduced weight gain and reduced feed conversion (29).

The farm also influenced the average weight in our study, indicating that farm-specific differences in flock size and management could have led to those results.

There was a seasonal influence on the average weight observed in our study, with the highest weight in fall and the lowest in spring. The highest ascites findings were also observed in winter and fall, therefore, a connection could be assumed. It has already been reported that ascites can be associated with a high growth rate (30) and there was an influence of the ascites findings on the average weight in our study. Additionally, ascites can be caused by insufficient oxygenation of the body, caused by the disproportionately small heart and lungs, because the modern broilers are bred for meat yield (31) and are thus not able to cope with the higher oxygen supply over the colder months (32).

An influence of neither the FWM nor the cumulative mortality was proven in our study, which is similar to the observations of Vasdal et al. (10), who also did not find an association between the growth rate and the FWM. The authors concluded that a low FWM does not necessarily result in a faster growth rate.

The overall condemnation percentage in our study was 1.48% (n = 107 flock cycles), with a minimum of 0.12% and a maximum of 4.63%. Of the variables we analyzed, the season, the production week of the parental flock, and the use of antibiotics significantly influenced the condemnation percentage. The average condemnation percentage of our study is higher than those reported by van Limbergen et al. (13) (1.23%), Alfifi et al. (14) (1.10%), and Nijdam et al. (33) (0.88%).

However, it is similar to the rates found by Kittelsen et al. (16) (mean 1.4%) and Santos et al. (30) (median 1.40%). The Federal Statistical Office of Germany recently stated that on average 2.10 % of the broilers slaughtered at German slaughterhouses in the year 2020 were not suitable for human consumption (33) and are thus higher than our findings.

Other studies identified the season as an influencing factor, similar to our study. Averós et al. (34) found a total percentage of carcass rejection of 0.77%, with the highest percentages in fall and spring. Salines et al. (12) found a total condemnation percentage of 1.04%, with the highest percentage in summer. They concluded that the high condemnation percentage in summer might be linked to heat, either during transport to slaughter or already on the farm (12). They also mentioned other possible influencing factors, such as the chick or feed quality (12). In our study, the highest condemnation percentages were found in fall and the lowest in winter and spring. In contrast, the mortality percentages were the lowest in fall and were high in winter and spring, and consequently, no influence was observed of either the FWM or the cumulative mortality on the condemnation percentage in our study. According to Vasdal et al. (10), a reduced condemnation percentage could be the result of poor flock uniformity, which is associated with a reduced growth rate and increased mortality rate and thus a poor general condition. An influence of the catching time on the condemnation percentage was not observed in our study. The condemnation percentage in total was also influenced by the production week of the parental flock in the same way and increased when the production week increased. This leads to the assumption that broilers from older parental flocks were more susceptible to infections which led to an increase in the condemnation percentage.

The DOA rate refers to birds that have died during their journey to the processing plant (35). In our study, the DOA percentage showed a mean of 0.17% (n=107 flock cycles) and was influenced by flock size, FWM, the use of antibiotics, and the interaction between antibiotic treatment and season. The mean DOA percentages reported in the literature vary greatly and range from 0.07% (15) to 0.46% (9), including reported percentages of 0.09% (16), 0.11% (36), and 0.30% (37). Flocks categorized as medium had a higher DOA percentage and the lowest DOA percentage was found in flocks that were categorized as small at the farm level. This could have been because the broilers in smaller flocks had less stress during catching and transport because especially the catching took less time. Chauvin et al. (36) and Nijdam et al. (9) also described the influence of the flock size on the DOA percentage. Bayliss and Hinton (35) described three influencing factors for the DOA percentage: health status of the flock, thermal stress during transportation, and physical injury. In our study, the FWM was shown to have an impact on DOA. With an increase in the FWM, the DOA increased as well. This could be linked to the health condition which, if it is poor, could cause high mortality during rearing and transport as the birds could not cope with the stress. Kittelsen et al. (16), on the other hand, found no differences at all in the DOA rates between flocks with low or high cumulative mortality, whereas according to Chauvin et al. (36), the DOA percentage is positively correlated with the cumulative mortality, the catching method, transportation, and weather conditions. On the contrary, Jacobs et al. (38) found a negative relationship with on-farm mortality, whereby the DOA percentage decreased by 9% with every 1% increase in on-farm mortality. We observed an influence of the catching time on the DOA rate, with higher DOA percentages found in the flocks caught in day time. This could be linked to the stress caused by heat and sunlight during the day, especially in the summer months, which the birds could not adapt to and therefore did not survive the transport. With regard to the seasons, several studies have reported an influence of the season on the DOA percentage but with different statements regarding the distribution. In the study by Averós et al. (34), the DOA percentage was highest in fall, followed by winter, spring, and summer. In the study by Petracci et al. (37), the DOA percentage was highest in summer, followed by winter, spring, and fall. Grilli et al. (39) reported a mean DOA percentage of 0.38% throughout the year, with the highest percentage in winter (0.52%) and the lowest in fall (0.22%). In our study, no influence of the season was observed.

Deep dermatitis was the main cause of condemnation (mean 0.63%, n=106 flock cycles) in our study and was influenced by the season and the production week of the parental flock. Dermatitis usually starts with an initial skin lesion and is followed by a secondary bacterial infection (40) with *E. coli* being the most prominent bacteria proven (41). Focal dermatitis is described as a thickening of the discolored skin, mainly unilateral, with brownish crusts. Plaques of yellowish fibrocaseous exudate could be observed on the subcutaneous tissue of the underlying skin and those lesions can mainly be found in the postventral region (41). The data evaluation of the Federal Statistical Office of Germany also name deep dermatitis as the main cause of condemnation in the year 2020 with deep dermatitis being the reason for 29.4 % of the condemned broilers (33). Similar to our results, Alfifi et al. (14) found skin disorders (scratches and dermatitis) to be the main cause of condemnation in broilers, with a prevalence of 0.24%. In addition, Salines et al. (12) found non-purulent cutaneous lesions in 20.00% of the broiler flocks and purulent lesions in 2.70%. The observed seasonal influence may have been due to the quality and moisture of the litter on the farm, a factor that is commonly associated with the season. The highest deep dermatitis findings were shown in summer, followed by fall, whereas deep dermatitis was far less frequent in winter. This could be because in the moist litter (commonly present in winter), the sharp claws of the broilers causing scratching are clogged by the litter. Tabler et al. (42) also described a higher incidence of gangrenous dermatitis in broiler flocks in summer and fall and less in winter and spring, which should be kept in check by constantly collecting the dead carcasses at the farm level. The findings of deep dermatitis in our study increased with the increasing production week of the parental flock. This could be explained by healthier birds from earlier production weeks of the parental flocks which were less vulnerable.

The other major cause of condemnation in our study was ascites (mean 0.53%, n=106 flock cycles). Ascites, the abdominal accumulation of fluid (43), was influenced by season, flock size, antibiotic treatment, and its interaction with season. On a national level, ascites also was found to be the second most frequent finding at German slaughterhouses and was the reason for 16.30 % of the condemned broilers in 2020 (33). Ascites is caused by an imbalance between oxygen supply and the oxygen required and results in hypoxia (44) and is the most common, non-infectious loss in the broiler industry (43, 44). Hypoxia leads to increased blood output and pulmonary hypertension, resulting in right ventricular hypertrophy, which leads to dilatation and failure of the right ventricle. The consequences are edema due to the increased blood pressure, from which the fluid leaks into the abdominal cavity (44). Ascites is known to have a genetic predisposition and can be liked to a fast growth rate (20). Other factors such as higher oxygen demand in the colder months of the year are also known to increase the risk of ascites (32). Alfifi et al. (14) recorded ascites with a prevalence of 0.22% in broiler flocks, whereas Gholami et al. (45) found cachexia as the most frequent cause of condemnation (0.15%) and ascites as a minor cause (0.03%). Salines et al. (12) found ascites in 0.10% of the flocks concerned, with generalized congestion being the most common finding (41.39% of the flocks assessed). All these reported percentages of ascites are far lower than our findings. Olkowski et al. (43) found ascites in 0.35% of the broilers condemned at slaughter and pointed out the associated risk of economic loss to the industry; the broilers with ascites are likely to die on the farm or during transport to the processing plant, and those reaching the slaughter line will be condemned. In their study, a slight seasonal trend was visible, with the incidence being highest during the colder months and lowest during summer (43). An influence of the season was also found in our study, with similar results, that is, the highest ascites percentages were found in winter, followed by the ascites findings in fall. Our results could be due to the higher oxygen demand during the colder months, as reported previously, to which the broilers' respiratory tract was not able to respond (44). Nevertheless, the ascites findings were influenced by the flock size, indicating the highest ascites findings in the flocks categorized as medium, whereas the lowest ascites findings were found in flocks categorized as small. This could be indirectly linked to the average weight, because broilers of medium flocks had the highest and small flocks the lowest weight. Therefore, a connection between ascites and the average weight can be assumed, as a relationship between ascites and growth rate has previously been reported (20, 44) and was also observed in our study within the ANOVA.

The less common causes of condemnation such as not suitable for production/general disease, hepatic changes, polyserositis, underdevelopment/emaciation, other pathologic findings/hematoma/injuries, and changes in color/odor/texture were influenced by several factors (season, cumulative mortality, stocking density, production week of the parental flock, the use of antibiotics and its interaction with season). Season was the only factor which influenced all of those dependent variables, except hepatic changes, which were only influenced by stocking density. These findings emphasize the need for maintaining good housing conditions during each season to keep the broilers in a healthy condition and thus maintain low mortality percentages. A high stocking density can be a reason for high levels of the various causes of condemnation because more birds per square meter can lead to an increase in infection pressure, stress level, and injury risk.

All broilers assessed in our study had been slaughtered at the same processing plant. Thus, the potential variance due to different assessment schemes that could lead to differences in the evaluation and documentation of slaughter results could be considered as low. Nevertheless, differences have been seen between the people who assess the birds during meat inspection (46), and also the Federal Statistical Office of Germany mentions, that although all animals are examined, some findings could not be evaluated and reported accordingly due to different recording and documentation possibilities in the slaughterhouses (33). Controlling and minimizing the use of antibiotics in Germany is of major importance, therefore, the German Federal Ministry of Food and Agriculture has implemented key points for a national antibiotic minimization concept for animal husbandry. In those, they describe that every farm with more than 10,000 broilers shall be part of the national plan to reduce antibiotic usage. Whenever an antibiotic treatment is performed, it must be documented and every 6 months those data must be sent to the HIT (central database) by the veterinarian. All data will be evaluated and whenever there is a higher usage of antibiotics than the average, special steps must be taken to improve the housing conditions and the wellbeing of the animals (47).

In our study, we furthermore considered the use of antibiotics to get an overview of the antibiotics used in the flocks observed and to investigate a possible influence on the outcomes of rearing and slaughter. One of the results we observed was that flocks with antibiotic treatment had a significantly lower cumulative mortality than flocks without antibiotic treatment. The most common causes of antibiotic treatment in our study were umbilical yolk sac inflammation (UYSI), enteritis (E), arthritis, and *E. coli* infection in broiler chicks. Antibiotic treatment was used when the mortality in a flock had increased significantly or an illness was diagnosed in the chicks. These results are in line with other publications considering antibiotic treatments of broilers (48). Other authors named neonatal septicemia to be one of the factors influencing the overall mortality (13), and the use of antibiotics would be required to control the infection. Considering the age at which the broilers were treated, 14 flocks were given antibiotics within the first week of the chicks' life, and all of these flocks were housed in spring. Treatments with Metaxol were done after the diagnosis of UYSI, whereas the other treatments done in the first week of the chick's life already started on the day the chicks were housed and after the diagnosis of enteritis. The flocks with a treatment of enteritis had a higher FWM (mean 0.69) than the flocks with antibiotic treatment because of a UYSI (mean 0.34). The Regulation (EU) 2019/6 gives information about the use of antibiotic treatments and states that antibiotic treatment must not be used routinely. It shall also not be used to compensate for poor rearing conditions and hygiene, lack of care, or poor farm management. Besides, it shall not be used to promote growth or increase yield. Prophylactic administration is only permitted in exceptional cases and only if it concerns individual animals or a limited number of animals and the risk of infection is high. In addition, the expected consequences must be severe. An antibiotic metaphylaxis is only permitted if, again, the number of animals is limited and the risk of spread of infection or infectious disease in a group of animals is high and no adequate alternatives are available (49). Two of the flocks in our study were treated with antibiotics three times and there were differences between the flocks with three antibiotic treatments with regard to the week the broilers were treated. The flock with antibiotic treatment in week 1, week 4, and week 5 had moderate first-week mortality of 0.54% (the average mean in total was 0.66%) and high cumulative mortality of 3.83% (the average mean in total was 2.74%). The flock with antibiotic treatments in week 3, 4, and 5 had first-week mortality mean value of 1.24% and cumulative mortality mean of 4.48%. Those numbers show that although health problems within the first weeks of the chick's life can be severe, diseases in the later course of the fattening period could have serious consequences.

An interaction between the antibiotic treatment and the season was visible for the FWM within the multivariate model, with differences between the season in which the treatment was done and also the number of treatments per flock. Most of the flocks that received antibiotics were treated during spring (18 of 30 flocks). Furthermore, only in spring two of the flocks were treated three times. An effect of the interaction between antibiotic treatment and season was observed on the average weight of the broilers, but for antibiotic treatment alone no effect was proven. The interaction between antibiotic treatment and season describes the seasonal differences between the flocks which had been treated and those which were not treated. Flocks without antibiotic treatment had a higher average weight, which could be linked to the better health status and a better appetite of the flocks, resulting in a better feed intake. After having discussed our results with the farm management of the two farms of our study, we were informed that the farm has noticed an increase in the flocks which needed to be treated within the first week. Therefore, it can be assumed, that our data collection was carried out during the period when the problems in the broiler fattening started and are less due to the time of the year.

The use of antibiotics during fattening also influenced the DOA percentage in our study. The mean DOA percentage in flocks without antibiotic treatment was 0.15%, whereas that in flocks that had been treated was 0.21%. The birds that had been treated were probably less resistant to stress during the transport, owing to their poorer state of health which made the previous treatment necessary.

Our analysis also showed that the records of ascites were influenced by the use of antibiotics as well as by the interaction between antibiotic treatment and season. There have been differences in the number of flocks that were treated between the seasons and this interaction was revealed within the model. Earlier infections in the broiler's life could have been a predisposing factor. *E. coli* infections, the main bacterial agent for antibiotic treatment in our study (Table 6), for example, can cause infections of the respiratory tract (55) and lead to damage. Ascites, a result of hypoxemia (44), could occur more easily as a result. In addition, Olkowski et al. (43) mention that a high percentage of broilers condemned at the slaughterhouse because of ascites can have other health issues such as cellulitis or cyanosis, which is not even reported, and cellulitis is often caused by *E. coli* infections (50), the most prominent reason for antibiotic treatments in the flocks observed. Thus, a connection between antibiotic treatment and ascites and its predisposing factors seems possible.

The interaction between antibiotic treatment and season for the DOA was also proved, which could be explained by less healthy birds, which had been treated during the rearing period. It can be assumed that the treated birds were less able to cope with the stress during catching and transport.

An influence from the interaction between antibiotic treatment and season was also found on the dependent variables ascites, polyserositis, other pathologic findings/hematoma/injuries, and changes in color/odor/texture with the highest findings being in winter in flocks that had been treated, except other pathologic findings/hematoma/injuries when the findings were lower in the flocks with antibiotic treatment. This shows that the seasonal differences between the antibiotic treatments had an effect on the slaughter results, and flocks without antibiotic treatments still were in better health at the time of slaughter.

Conclusions

The presented multivariate analysis revealed several factors that can affect the mortality of broilers during the rearing period, their slaughter weight, and the causes of condemnation recorded at the processing plant. Cumulative mortality was influenced by FWM, antibiotic treatment, and the farm. FWM was influenced by flock size, the interaction between antibiotic treatment and season, and the production week of the parental flock. The influence of antibiotic treatment on FWM could be a result of an infection in the flock, which in many cases entails high mortality, in our study especially within the first 7 days of the chicks' life considering the differences between the seasons. Therefore, we recommend practicing special care in chick management to prevent increased losses during the fattening period and excellent hygiene to protect the health of the chicks. The average slaughter weight was influenced by the season, the stocking density, the flock size, and the farm as well as the interaction between the use of antibiotics and the seasons. The condemnation percentage was influenced by the season, the production week of the parental flock, and the use of antibiotics, whereas the DOA percentage was influenced by the FWM, the flock size, the use of antibiotics during fattening, the interaction between antibiotic treatment, and season and, in addition, by the catching time. We assume that the flocks with high FWM and the flocks that had been treated with antibiotics included animals that were less resistant to stress owing to poorer health status. Thus, they did not adapt well to the transport. The most prominent causes of condemnation (deep dermatitis and ascites) both were influenced by the season. Deep dermatitis was additionally influenced by the production week of the parental flock, whereas ascites was additionally influenced by the flock size, the use of antibiotics, the interaction between antibiotic treatment and season, and also by the average weight. Season, followed by the interaction between the use of antibiotics and season and stocking density were the independent variables, which mainly influenced the condemnation causes in our study. Although the rearing conditions are supposed to be consistent in each barn and flock throughout the whole year, there seem to be differences regarding the seasons which result in those outcomes. The stocking density could be an influencing factor because, with its increase, the infection pressure can increase similarly. The influence of antibiotic treatment alone or its interaction with the season has been observed several times in our study. This influence should be reduced by keeping excellent rearing conditions and feed and water of good quality to meet the national standards. Nevertheless, antibiotic treatment, if necessary, should be done as early as possible because infections in the flocks can increase the condemnation percentage, can lead to financial losses, and are of concern from an animal welfare perspective.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because the study exclusively included data generated after slaughter without any procedure or intervention on living animals. The data used is also generated within normal husbandry conditions in livestock.

Author contributions

LD collected most data and transferred them to an Excel table. AJ checked the data and supplemented and completed them, conducted the statistical analysis, and prepared the first draft of the manuscript. HL commented on the previous versions of the manuscript. All authors approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dairy sheep and goats prefer the single components over the mixed ration

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Mixed rations provide ruminants with a balanced diet by aiming to prevent selective feeding. However, this is a natural behavior of sheep and goats based on their dietary needs and the nutritional properties of feedstuffs. Therefore, the present study investigates non-lactating dairy sheep's and goats' acceptance of a mixed ration when it is offered as choice next to its single components. Because all offered feeds were of comparable nutritional value, the animals were expected to not show a particular preference. Twelve pairs of sheep and goats each, were offered three different feeds simultaneously for 5 replicate days. Two feeds consisted of a single component, hay (H) or grass-silage (G) of similar nutritional value. The third feed was a mixed ration (M) including both single-feed components in a 50:50 dry matter (DM) ratio. Feeds were offered *ad libitum* twice daily. The animals' intake of each feed was recorded at six time points per day by weighing the leftovers. Feed preference was expressed as the natural logarithm of the ratio of the intake of the single component to the intake of M and analyzed using linear mixed-effects models. Additionally, the animals' first choices after gaining access to the feeds were recorded at each weighing event and analyzed using an item response tree generalized mixed-effects model. The sheep's average daily DM intake was 59 (±11)% G, 26 (±10)% H, and 15 (±10)% M (mean ± standard deviation). Goats consumed an average of 56 (±13)% G, 37 (±12)% H, and 7 (±6)% M daily. Both species preferred the single components to M in all observation periods. The proportions of the three feeds consumed differed throughout the day and between species. For both species, the estimated probability that an animal chooses a single component over M first was over 94% at all time points. These results show that, contrary to our expectations, non-lactating dairy sheep and goats prefer single components over a mixed ration of the same components and similar nutritional value. This might be caused by the animals seeking to diversify their feed throughout the day independent of apparent nutritional values and/or because sensory properties of the single components, indicating palatability, are relevantly reduced by mixing.

KEYWORDS
feed preference, ruminant, sheep, goat, silage, feed choice

Introduction

The use of mixed rations played a major part in the intensification of beef and dairy cattle production in recent decades (1). Mixed rations are fed as partial mixed rations (PMR) or total mixed rations (TMR). In PMRs, usually, roughage feed components are mixed and other components, such as concentrates, are offered separately. TMRs, on the other hand, contain all ingredients of the diet, including minerals and concentrates. Feeding livestock mixed rations is labor-efficient, reduces feed refusal, and provides nutritional advantages for the animals (1). Mixing components allows to combine less palatable feedstuffs with more palatable ones into a balanced diet and to easily adapt to various production levels (1). Mixed rations also enable all animals in a herd to access the same feed by preventing individual animals from monopolizing access to preferred feedstuffs (2) such as concentrates (3). Additionally, mixed rations reduce sorting for feed components in cattle (1), resulting in more consistent feed quality over time. This increases feed intake, especially for animals that reach the feed later than others in the herd (4), thus increasing animal productivity and feed efficiency (5).

In small ruminants (sheep and goats), the use of mixed rations is not yet as widespread as in cattle. However, the general worldwide trend toward fewer farms with larger herd sizes suggests that this labor-efficient feeding system will also be increasingly used for small ruminants. The effects of feeding mixed rations on productivity in small ruminants have been investigated, but the results are less consistent than for cattle. Monzón-Gil et al. (6) demonstrated that TMR feeding increased feed intake and milk yield in goats compared to single component feeding. Görgülü et al. (7) found that goats freely choosing the ratio of feed components (of the compared TMR) showed higher dry matter intake and higher milk yield than TMR-fed goats, although milk production efficiency was better on the TMR diet. In contrast, Yurtseven et al. (8) found that in sheep TMR feeding had no effect on milk production performance compared to free-choice feeding with the feeds of the compared TMR.

To better understand the effects of mixed-ration feeding in small ruminants, it is necessary to consider these animals' distinct feeding behavior. The ancestors of sheep and goats evolved predominantly in harsh environments and thus developed very selective foraging and feeding behaviors as an adaptation to seasonal and local variations in the availability of feed plants (9). Domestic sheep and goats kept in natural and semi-natural environments use selective browsing to adapt their intake to their nutritional needs (9–11). Sheep and goats also sort components of a feed (12) and choose among different feeds in indoor feeding conditions according to the varying nutritional needs of their current physiological stage (12). Therefore, it is unclear whether mixed rations are appropriate for sheep and goats as these rations are explicitly designed to limit selective feeding (13).

Previous studies have found that sheep and goats select their feed based on nutritional aspects in order to obtain a diet that meets their nutritional requirements. For instance, sheep and goats have both shown a preference for forages with higher organic matter digestibility and lower fiber content, preferring, for example, leafy grass hay to mature grass hay or straw (14). In short-term preference tests (3 min sessions), goats' feed choices were more influenced by the type of starch than by forage-to-concentrate ratios; they preferred starches that degrade rapidly in the rumen to those that degrade slowly (15). In a three-week feeding experiment, sheep ate more feeds supplemented with $NaHCO_3$ than unsupplemented feeds (16). Goats have also been shown to adapt their concentrate intake based on its crude protein concentration, eating less soybean-based concentrate (which is high in crude protein content) than chickpea-based concentrate (which is lower in crude protein), leading to a consistent percentage of crude protein intake in the total diet (17).

Additionally, small ruminant adapt their feed intake and choices based on what feeds they have already consumed. It is assumed that they do this by monitoring the current condition of the rumen (18). For example, sheep's consumption of low-energy-density feeds depends on the carbohydrate sources of other feeds consumed (16). Thus, although small ruminants prefer energy-dense feeds (19), they apparently substitute their diet with feedstuff higher in fiber contents if necessary to balance the ruminal pH (20). This might explain why free-choice-fed goats prefer different feeds at different times of day (7). When foraging in natural and semi-natural environments both species prefer different plant species when the available variety is not restricted (11). But for harvested feeds of restricted number of options [six forages (14)] and for artificially flavored feeds (21) sheep and goats show similar preferences.

Based on the studies described above, one would expect that sheep and goats will not show a preference for a particular feed if all offered feeds meet the animals' nutritional requirements and are comparable in terms of properties such as energy density and fiber content. Therefore, the aim of this study was to assess non-lactating dairy sheep's and goats' acceptance of a mixed ration when the single components of that ration, grass silage and hay, are offered at the same time. All three feeds (mixed, grass silage, and hay) had similar nutritional value and met the animals' nutritional needs. We therefore hypothesized that, on average, all three feeds would be consumed by both species in similar amounts regardless of the time of day.

Materials and methods

Animals and housing conditions

The experiments were conducted in October 2020 at the Agroscope Research Station in Ettenhausen, Switzerland. The sample included 24 female dairy goats (10 Saanen, 11 Chamois Colored goats, 3 crossbreeds) and 24 female dairy sheep (20 Lacaunne 4 East Friesian sheep). All animals were 3 years old and had never been lactating or pregnant. At the start of the experiment, the mean body weight of the goats was 67.5 (standard deviation ±6.9) kg, and the mean body weight of the sheep was 78.4 (±7.9) kg. During the experimental phase, the goats and sheep gained an average of 1.94 and 1.19 kg, respectively.

Prior to the experimental phase, the sheep and goats were kept in the same stable in an outdoor climate with one pen for each species. The goat pen had a total area of 53 m^2 (13.6 × 3.9 m), including a straw-bedded deep litter area of 40 m^2 and an elevated feeding area, 0.95 m wide, along the long axis of the pen. The deep litter area was equipped with three benches (2.4 × 0.62 m; height: 0.6 m) and three round tables (diameter: 1.1 m; height: 0.8 m). The sheep pen had a total area of 42 m^2 (11.7 × 3.6 m) with a deep litter area of 33 m^2 and an elevated feeding area, 0.8 m wide, along the long axis of the pen. Each pen had three drinkers for *ad libitum* access to water and one mineral supply. Feed troughs with a palisade feeding fence (35 and 40 cm feeding space per animal for goats and sheep, respectively) were placed along the entire long axis of each pen.

All animal care and experimental procedures were performed in accordance with the relevant legislative and regulatory requirements and the ASAB/ABS Guidelines for the Use of Animals in Research (22). The Cantonal Veterinary Office, Thurgau, Switzerland (Approval No. TG10/18–30902) approved all procedures involving animal handling and treatment.

FIGURE 1
Top view of the feeding trough in the experimental pen (for sheep or goat pairs) with three plastic feed containers per feeding place filled with either grass silage (G), hay (H), or the hay-grass silage mixed ration (M). **(Top)** the animals have access to the feed. **(Bottom)** access to the feed is blocked while the containers are weighed and to measure the animals' first choice after access is given.

Experimental setup and procedures

Experimental pens

The experiments were conducted in a separate outdoor climate stable consisting of four sheep and four goat pens, each large enough to house two animals (mean daily temperature: 9.4°C, BAFU/EUA, MeteoSchweiz). Each pen was 2.4 m × 3.5 m and included an elevated feeding area with two places equipped with a trough. Two pens shared one drinker with *ad libitum* access to water. The two feeding places were separated by a solid wood wall (1.4 m × 0.95 m) to minimize agonistic interactions (23), but allowed visual contact in the area above the trough (Figure 1). The litter area was bedded with sawdust. The goat pens were additionally equipped with round wooden tables for climbing and elevated resting (diameter: 1.1 m; height: 0.8 m).

Feeds and habituation

Three feeds were used in the experiments. Two of these were single component feeds: chopped hay (H) and chopped grass silage (G) with cutting lengths of ∼3–4 cm. The third feed was a mixed ration (M) consisting of the same H and G mixed in a 50:50 dry matter (DM) ratio. G and H had similar protein, fiber, and calculated energy content, as well as a similar botanical composition and were both harvested at the beginning of the flowering stage (Table 1). Both met the nutritional needs of non-lactating sheep and goats (25). To make M, grass silage was added to the mixer wagon (Jaylor Model 5100 Self Propelled, Canada) first. Hay was then added, and the two were mixed for ∼10 min. The mixer wagon did not include knives to avoid structural changes on the feeds. M was freshly prepared every day.

The animals were familiar with the three feeds from previous experiments (between experiments, animals received uncut hay *ad libitum*). Nevertheless, a habituation phase was conducted prior to the start of the experiment to avoid any neophobic reaction to the feeds (26). Ten days before the experimental phase began, all animals received one of the three experimental feeds *ad libitum* in their group stable; the three feeds were switched daily. In total, the first experimental group (see paragraph Test procedure) received G on 3 days, H on 3 days, and M on 4 days. The second and third group received these feeds for twice and three times as many days as the first group, respectively.

Test procedure

The experiment lasted five replicate days for each experimental group. The animals were tested in pairs as stress due to isolation can inhibit feed intake (27). The eight experimental pens were used simultaneously, and the animals were divided into three experimental groups, each group including four pairs of goats and four pairs of sheep.

During the experiment, 50% of the daily ration was offered at 09:00 and 50% at 15:00 via topping up (Table 2). Each animal was offered the three feeds simultaneously in separate plastic containers (28 × 34 × 22 cm). These containers were placed next to each other inside the feeding trough (Figure 1). The positions of the three containers were switched daily in a semi-random order to avoid confounding due to a possible side preference. Each of the three feeds was offered at 100% of the expected daily DM intake, which was estimated using the maximal daily DM intake of similar feeds by the same animals during previous experiments. The overall offer therefore comprised 300% of the animals' anticipated intake.

TABLE 1 Chemical and botanical composition of grass silage (G), hay (H), and the mixed ration (M).

	Unit	G	H	M	Need goat[1]	Need sheep[2]
Dry matter (DM)	% of fresh weight	31.4	91.3	50.7	–	–
Organic matter	g/kg DM	901	915	907	–	–
Crude protein	g/kg DM	121	108	118	91	81
ADF	g/kg DM	254	263	253	>200[#]	>200[#]
NDF	g/kg DM	428	506	478	>410[#]	>410[#]
NEL*	MJ/kg DM	5.5	5.3	5.4	5.1	4.5
APDE*	g/kg DM	69	81	76	36	32
APDN*	g/kg DM	76	68	75		
Ryegrass	%	75	80–90			
Clover	%	20	10–20			
Herbs	%	5	< 5			

ADF, acid detergent fiber; NDF, Neutral detergent fiber; NEL, Net energy for lactation; APDE, Absorbable protein at the duodenum limited by rumen-fermentable energy; APDN, Absorbable protein at the duodenum limited by rumen-fermentable nitrogen.
[1] goat with a mean weight of 67.7 kg and mean daily DM intake of 1.5 kg; [2] sheep with a mean weight of 78.1 kg and mean daily DM intake of 1.9 kg; [*] calculated according to Agroscope (2021); [#] recommended 20% of daily DM intake ADF and 41% NDF (24) (see comments in table).

TABLE 2 Observed dry matter (DM) quantities of grass silage (G), hay (H), and the mixed ration (M) offered and consumed per day and animal.

	Unit	G	H	M	Total
Goats					
DM offered	kg	1.2	1.4	1.0	3.6
Mean DM intake (±SD)	kg	0.9 ± 0.3	0.5 ± 0.2	0.1 ± 0.1	1.5 ± 0.3
Corrected	g/kg LW $^{0.75}$	36.0 ± 12.1	23.1 ± 6.5	5.0 ±.7	64.1 ± 11.9
Refusals	%	39	55	90	59
Proportional intake	%	57	36	7	100
Sheep					
DM offered	kg	1.4	1.9	1.2	4.5
Mean DM intake (±SD)	kg	1.1 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	1.9 ± 0.3
Corrected	g/kg LW $^{0.75}$	40.9 ± 8.5	18.1 ± 7.5	11.1 ± 6.9	70.1 ± 7.9
Refusals	%	43	66	77	59
Proportional intake	%	59	26	15	100

Feed preference recordings

The animals' intake of the three feeds and first feed choices were recorded. Intake was recorded for the animal pairs, and first choice was recorded for each individual. The feed containers were weighed seven times a day at 09:00, 10:00, 12:00, 15:00, 16:00, 18:00, and ~08:30 the following day. Intake of each feed was then calculated for the following time periods: 09:00 to 10:00, 10:00 to 12:00, 12:00 to 15:00, 15:00 to 16:00, 16:00 to 18:00, and 18:00 to 8:30 am the following day. In the following sections, the periods 09:00 to 10:00 and 15:00 to 16:00 are referred to as the "main meals." These periods correspond to the first hour after feeding, where most feed is consumed per unit of time (28). The time periods from 10:00 to 12:00 and from 16:00 to 18:00, referred to as the "second periods," are used to compare to main meal results. Feed intake is expressed as grams of DM per kg metabolic life weight (g DM/kg LW$^{0.75}$). To approximate intake per individual, the fresh matter intake recorded per pair was converted to its DM equivalent and divided by the sum of the pair's LW$^{0.75}$.

The animals' first choices of feed were recorded at each time point of weighing the containers as follows. While the containers were weighed, the animals' access to the trough was blocked with a wooden barrier (Figure 1). After the containers were placed back in the trough, the barrier was removed. The first choice was recorded as the feed that was ingested first after the barrier was removed. Individuals that did consume one of the feeds within 1 min after the barrier was removed were recorded as "participating" in the first choice test. Accordingly, individuals that did not do so were recorded as "not participating."

Feed analyses

Samples of the fresh M were taken daily, and samples of the H and G were taken on days 1, 3, and 5 of the experimental phase. Samples were dried at 60°C for 48 h to calculate the dry matter content as percentage of fresh matter. For the subsequent chemical analyses, dried samples were pooled per experimental group and ground to pass a 1-mm screen (Brabender rotary mill; Brabender GmbH & Co. KG, Duisburg, Germany). Feed samples were analyzed for exact dry mass content by heating at 105°C for 3 h (prepASH, Precisa Gravimetrics AG, Dietikon, Switzerland) and then incinerating at 550°C until a stable mass was reached to determine the ash content according to ISO 5984_2002. Organic matter was calculated by subtracting the ash content from the dry matter content. The Neutral detergent fiber (αNDF; ISO 16472:2006) and acid detergent fiber (ADF; ISO 13906:2008) contents were analyzed with a fiber analyzer (Fibretherm Gerhardt FT-12, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and were expressed without residual ash. Neutral detergent fiber (αNDF) was determined after treatment of the sample with heat stable amylase and sodium sulfite and expressed without residual ash after incineration at 600°C for 3 h.

Statistical analyses

For the statistical analyses and data visualization, we used the open-source software R version 4.2.0 (29). The preference between the three offered feeds was investigated by a log ratio transformation of the feed intake data to avoid the complications otherwise associated with such compositional data (30). A small positive values (0.01 g DM/kg LW$^{0.75}$) was assumed for apparent zero intake to allow the calculation of log ratios (30). The natural log ratios of H and G to M were analyzed using linear mixed-effect models, which was estimated using the *lmer* function of the lme4 R package (31). The model formula in *lme4* syntax is as follows:

$$\log(\text{H or G / M}) \sim 0 + \text{Species} : \text{Period} + (1 \mid \text{Group/Pair})$$

The model includes an intercept for each period individually for both species as the fixed effect (0 + Species:Period). Furthermore, a random intercept for pair nested within group (1 | Group/Pair) to account for repeated testing of the same animal pair over replicate days and for the potential effects of group affiliation. Only the main meal and second periods were included. The other periods (10:00 to 12:00 and 18:00 to the next day) were excluded as their lengths varied and they included overnight.

The data on the animals' first choice of feed was analyzed using an item response tree model [IRTree, (32)]. Therefore, the data was encoded as a binary response tree with three nodes (Figure 2). The first node indicated participation (1: yes; 0: no), the second node indicated whether the animal chose a single component or M (1: G or H; 0: M), and the third node indicated whether the animal chose G or H (1: G; 0: H). The IRTree model was estimated as generalized linear mixed model (GLMM) with a binominal response and a logit link function using the *glmer* function from R package lme4. The model formula in *lme4* syntax is as follows:

$$\begin{aligned} &\text{value} \sim 0 + \text{Node} : \text{Species} \\ &+ \text{Node} : \text{Species} : \text{AmPm} : \text{TimeAfterFeeding} \\ &+ (0 + \text{Node} \mid \text{Group/Pair/Individual}) + (1 \mid \text{Obs}) \end{aligned}$$

The fixed effects in this model includes an individual intercept for each node for the two species (0 + Node:Species) and an individual slope for the time after feeding for both species separately for the time after the morning and after the afternoon feeding (Node:Species:AmPm:TimeAfterFeeding; the binary variable "AmPm" indicates morning or afternoon). Furthermore, we specified a random intercept for each node for the individual, nested within pair, nested within group (0 + Node | group/pair/individual). This accounts for repeated testing of the same individual and the potential effects of pair and group association. Finally, a random intercept for the observation (1 | Obs) is included to ensure that the binary responses at the three nodes that belong to the same observation are considered to share the same variance. For a detailed discussion of data encoding and model formulation for IRTree GLMMs, see López-Sepulcre et al. (32).

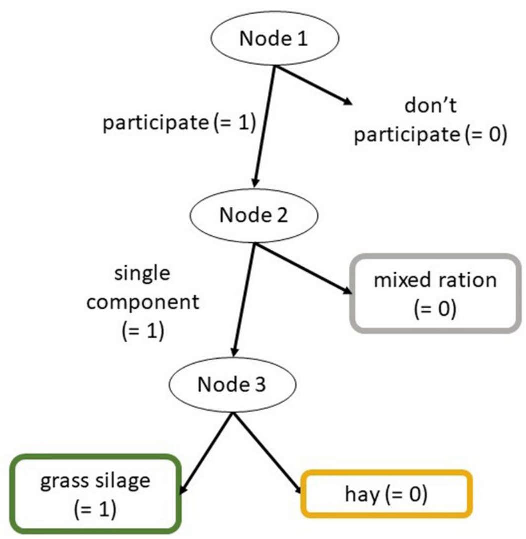

FIGURE 2
Binary response tree; IRTree model for analyzing first feed choices.

In order to investigate the differences between goats and sheep and between different periods, we tested linear contrasts for the fixed effects in the different models using the *glht* function of the R package multcomp (33). The significance of fixed effects and contrasts were assessed using bootstrapped 95% quantile confidence intervals ($CI_{95\%}$), which were determined via parametric bootstrapping as implemented in *bootMer* (10,000 bootstraps, R package lme4). This provides more reliable results than *p*-values based on Wald statistics (31). A significant difference from a null value (typically 0) at the 0.05 level is indicated when the $CI_{95\%}$ does not include the null value. Additionally, bootstrapped 95% quantile confidence bands for figures showing population-level fit, as is described by the fixed effects, were obtained using the *predict.MerMod* function (parameter re.form = ∼ 0; lme4 package) and the *bootMer* function for parametric bootstrapping (10,000 bootstraps). To overcome the prohibitive computational burden, the IRTree GLMM were refitted to bootstrap samples using the parameter nAGQ = 0 (a faster but less precise method of parameter estimation).

Results

Feed intake

The total mean daily intake (± standard deviation) per individual for goats and sheep was 64.1 ±11.9 g DM/kg $LW^{0.75}$ and 70.1 ± 7.9 g DM/kg $LW^{0.75}$, respectively. The observed mean daily DM intake of M per individual was 5.0 ± 3.7 g DM/kg $LW^{0.75}$ for goats and11.1 ± 6.9 g DM/kg $LW^{0.75}$ for sheep. The proportion of M in the DM intake varied from 0 to 17.7% in goats and from 5.3 to 26.1% in sheep over the different measured time periods. Goats did not eat M at all during the main meals (Table 2; Figure 3).

The results of the mixed effects models for preference as log intake ratios are shown in Figure 4. Log ratios > 0 indicate preference for the single component over the mixed ration, and values < 0 indicate the reverse. Both species ate more G and

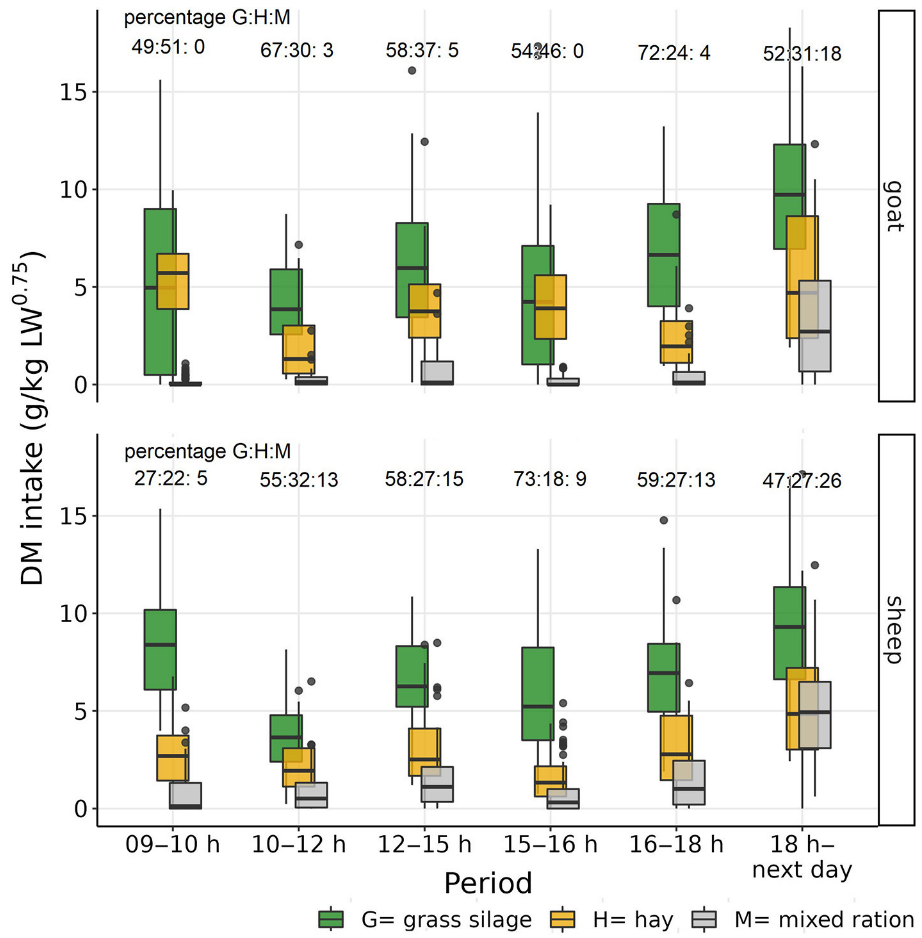

FIGURE 3
Boxplot of observed individual goats' and sheep's dry matter (DM) intake of the three experimental feeds, grass silage (G), hay (H), and the mixed ration (M), during the different observation periods.

H than M both overall and during the two main meals and second periods (all $CI_{95\%} > 0$). Comparing the preferences between the species, during the second periods, goats preferred G to M more than sheep (goat-sheep contrast +1.41; $CI_{95\%}$ 0.48–2.39) while there was statistically not sufficient evidence for such a between-species difference during the main meals (+0.09; $CI_{95\%}$ −0.87 to 1.02). The preference of H to M was higher for goats than sheep; this difference was most apparent during main meals (+2.37; $CI_{95\%}$ 1.37–3.33) and less pronounced during the second periods (+0.96; $CI_{95\%}$ 0.02–1.98). Comparing the two types of periods within each species, goats showed a higher preference for H to M during main meals than during second periods (main meal-second period contrast +1.98; $CI_{95\%}$ 1.39–2.57) while there was statistically not sufficient evidence for such difference in their preference of G to M (−0.11; $CI_{95\%}$ −0.64 to 0.42). Sheep, on the other hand differed between main meals and second periods in their preference of G to M (+1.22; $CI_{95\%}$ 0.68–1.17) and statistically less supported also in their preference for H to M (+0.58; $CI_{95\%}$ −0.02 to 1.17; Figure 3).

First choice

The overall observed rate of participation in the first-choice test (i.e., the animals started eating one of the feeds within the first minute after regaining access to the trough) was 51.3 and 51.9% for sheep and goats, respectively.

The results of the IRTree model are presented in Figure 5. In the morning, the estimated probability of participation (node 1) for both species decreased from over 0.8 at feeding ($CI_{95\%}$ 0.74–0.90 for goats; $CI_{95\%}$ 0.70–0.87 for sheep) to around 0.5 one hour after feed delivery and to below 0.28 three hours after feed delivery. Similar declines were observed in the afternoon, but initial participation was lower during the afternoon main meal ($CI_{95\%}$ 0.53–0.76 for goats; $CI_{95\%}$ 0.59–0.81 for sheep)

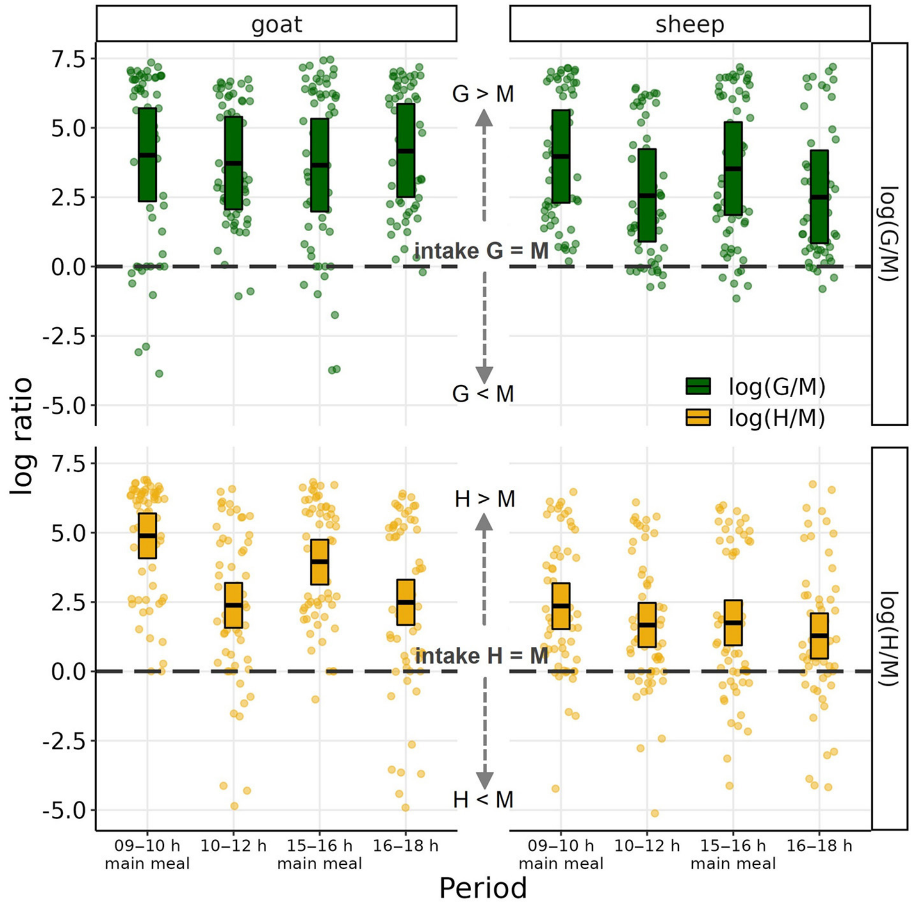

FIGURE 4
Log ratios of dry matter (DM) intake of grass silage (G) to mixed ration (M) and of hay (H) to M by goats and sheep during the four observation time periods. Boxes represent population-level mean log ratios with bootstrapped 95% confidence intervals as estimated by the linear mixed-effects models. Dots represent observed log ratios.

than in the morning. The experimenters observed that three hours after feed delivery (at 12:00 and 18:00), many animals were ruminating.

When the animals participated in the first-choice test, the probability to choose a single component over M (node 2) was estimated over 0.86 throughout the day for both species (all CI_{95} > 0.67; Figure 5), indicating a clear preference (>>0.5) for the single component. This probability was slightly higher for goats than for sheep (goat-sheep contrast on logit-scale: +1.98; CI_{95} 0.71–12.34). No sufficient evidence was found that this choice would differ between morning and afternoon nor over time after feed delivery (Figure 5).

When deciding between the two single components G and H (node 3), goats were more likely to choose H at the initial feeding (09:00, 15:00) as the probability that they would choose G was <0.5 (0.17, $CI_{95\%}$ 0.11–0.32 at 09:00 and 0.15, $CI_{95\%}$ 0.08–0.29 at 15:00; Figure 5). However, the probability to choose G increased with time after feeding (slope on logit scale: +0.68, $CI_{95\%}$ 0.39–0.88) up to that there was no statistical support for a preference between G and H anymore three hours after feeding ($CI_{95\%}$ 0.34–0.80 at 12:00; $CI_{95\%}$ 0.38–0.80 at 18:00). For sheep, there was no sufficient statistical support for a first choice preference between G and H at any of the sampling time points (all $CI_{95\%}$ include 0.5) but similar to goats an increase of the probability to choose G with time after feeding was indicated (slope on logit scale: +0.42, $CI_{95\%}$ 0.16–0.68; Figure 5).

Discussion

This study has investigated whether non-lactating dairy sheep and goats will eat a mixed ration (M) of hay (H) and grass silage (G) when the single components of the mixed ration were offered simultaneously. Although the proportions of feed intake of the three feeds varied throughout the day and differed between the two species, the animals showed a clear preference for G and H and barely consumed M. The same pattern was also seen in the choice of feed consumed first after access to the trough was given.

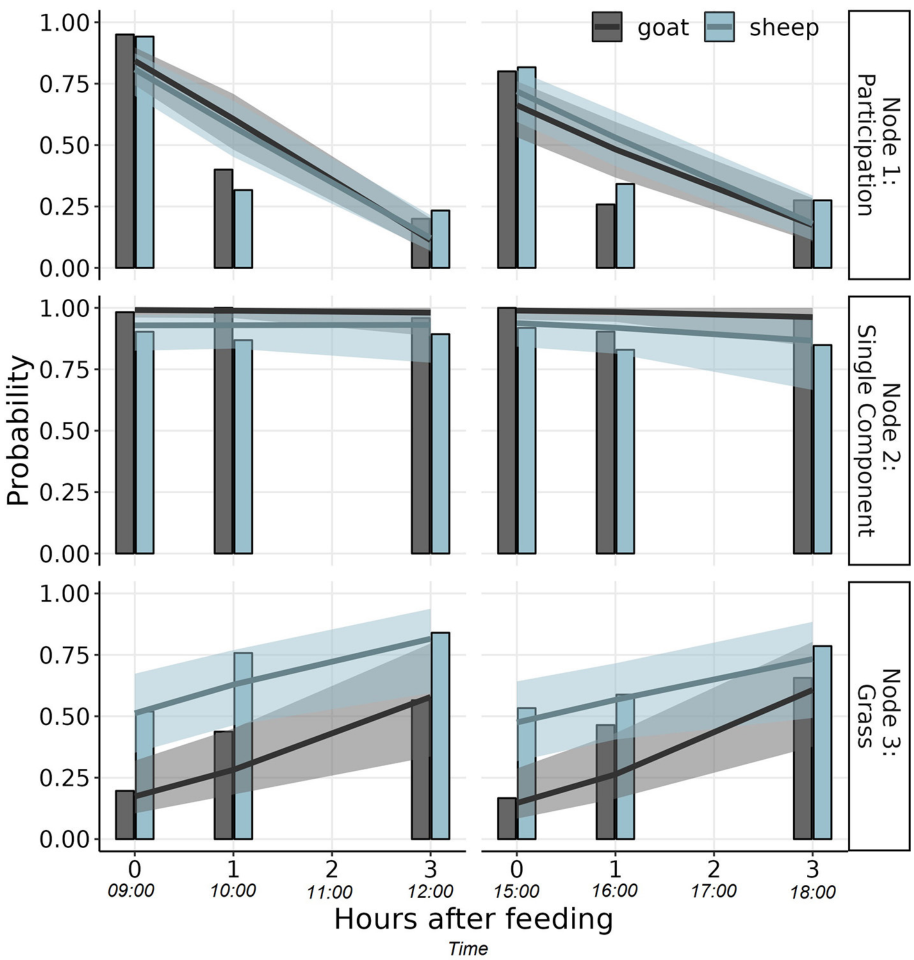

FIGURE 5
Probability that an animal would choose a feed (i.e., participate) within the first minute after gaining access to the trough (node 1), that the animal would choose a single component (hay or grass silage) over the mixed ration (node 2), and that it would choose grass silage over hay (node 3) at six time points for goats (black) and sheep (blue). Solid lines represent population-level (i.e., described by fixed effects), means with 95% confidence bands as estimated using the IRTree GLMM (shaded area).

Prior studies of sheep and goats in indoor feeding conditions have explained feed preferences and selection by nutritional value [goats: concentration ratios and starch types (15), type of protein concentrates (17); sheep: energy density (16); sheep and goats: chemical composition of forages (14)]. In the present study, all three feeds offered had comparable nutritional values and adequately met the nutritional needs of the tested animals (25). Still, in our study, both species refrained from eating the mixed ration, clearly preferring the single-component feeds. Apparently, small ruminants regulate their feed intake based on additional factors not related to the nutritional value of the feed.

A possible explanation for the animals' preference for G and H over M could be the ratio at which the two components were offered in the mixed ration (G:H 50:50). Goats have been shown to select ratios of feed components different from that of a mixed ration calculated to optimally meet their mean nutritional requirements (34). In the present study the nutritional contents of the three feeds did not differ and can therefore not be the reason for the low relative intake of M compared to the single components. The ratio of the two components in M also seem unlikely to be the main reason for the general avoidance by all animals, as at least the goats consumed H and G in a 50:50 DM ratio during their main meals (09:00 and 15:00), which was the same DM ratio of the offered mixed ration. Further studies on different mixing ratios could reveal whether a higher proportion of the one or the other components would increase the acceptance of the mixed ration next to its single components, or whether it is the process of mixing that caused the low relative intake.

The animals might have avoided M because its fixed ratio of the two feeds did not allow for a variation among meals. Görgülü et al. (7) found that free-choice fed goats showed a daytime dependent intake of the different offered feeds and grazing sheep selected different grass species (clover and ryegrass) in the morning than in the afternoon (35). They concluded that ruminants base their feed choice during main meals on attributes that indicate high nutritional value (e.g., rapidly degradable starch). Through post-ingestive feedback (19), they then balance their ruminal milieu during secondary meals by eating different types of feeds, such as those that are high in fiber. Nevertheless, in our study, despite the feeds' comparable nutritional values, the sheep and goats selected different ratios of the feeds throughout the day. Apparently, foraging and eating on a high variety of different plants is a strong behavioral adaption (36, 37) that evolved to ensure a balanced diet (13, 38) and will be performed even if it is not necessary to ensure an adequate supply of nutrients. For example, Scott and Provenza (39) found that lambs diversified their diet by choosing differently flavored rations (apple, anise, fresh forage), even though the rations had similar nutritional values. In another study sheep and goats showed to be sensitive to artificial flavors when choosing feeds as well (21). Since other attributes of the feed than its nutritional contents apparently play a role in sheep's and goats' feed preferences, the present study raises the question of whether mixed rations provide a suitable diet from a welfare perspective, as the mixing itself seems to reduce the palatability.

Our results are consistent with the model developed by Baumont et al. (20) to explain forage intake in small ruminants. This model suggests that the sensory properties of a feed impact the animal's motivation to eat and that the nutritional value of the feed regulates quantity by providing feedback about satiation. Because the feeds we offered had similar nutritional values, sensory properties must be responsible for the animals' feeding behavior in the present experiment. The physical form of feed (e.g., particle size, resistance to fracture, pellets), moisture, smell, and taste have been suggested as the sensory attributes of feeds that impact feed preferences (20). Maybe certain specific sensory attributes of H and G (e.g., dry vs. wet, sour vs. not sour, crispy vs. soft) were substantially diminished or diluted through the process of mixing, resulting in less preferred forms of these attributes. This could explain why the animals avoided M, when they had the choice for H and G but ate normal amounts of M when it was the only feed available (like during the habituation phase). However, only one kind of mixed ration was tested in this study and further investigations are needed to gain a more generalized understanding of small ruminants' acceptance of mixed rations.

The animals' choices of feed consumed first within the 1 min after they regained access to the trough were consistent with their overall feed intake. Both species rarely chose M, and overall intake of M was very low. Sheep's overall intake of G was more than that of goats, and sheep were also more likely than goats to choose G first rather than H. In another study of goats, Abijaoudé et al. (15) found that the feed with the highest daily DM intake was also the preferred one in 3-min choice tests. More recently, Scherer et al. (40) showed that goats' initial feed intake during the first 3 min of a choice-feeding experiment strongly predicts the DM intake over 3 h of feeding. Although it remains unclear which attributes of a feed impact the first choice, the present study confirms that sheep and goats seem to be able to rapidly distinguish the feeds on offer and that their first choice is a good indicator of not only short-term intake (3 h) but also total daily feed intake. Very little research has compared short-term and long-term preferences in ruminants, although Meier et al. (26) mentioned that this distinction could be important in feed choice experiments.

Several aspects that could influence the findings of the present study need further investigation. For instance, all our experimental animals were non-lactating, non-pregnant females of only 2 breeds per species. The external validity of our results are therefore limited. However, a previous study of goats found that feed preference was not impacted by the physiological stage [pregnancy or early or mid-lactation (37)]. Secondly, although the botanical composition and stage of harvest of G and H were similar and the feeds we offered had comparable compositions of macronutrients, their compositions of micronutrients, such as minerals, could have differed. These and other factors could have influenced feed choice between H and G. However, this limitation of the present study does not compromise the main result, that single components were clearly preferred over the mixed ration. Thirdly, on commercial farms, more than two feed components are often used in different mixed rations (41). In order to evaluate whether dairy sheep and goats prefer single-component feeds to mixed rations in general, numerous different mixed rations would have to be tested against their respective feed components. Of particular interest would be the animals' acceptance of total mixed rations, which contain all the components needed to optimally supply the animals' nutritional needs, including minerals and salts as well as concentrates. A consistent clear preference for single components over different mixed rations would indicate that this foraging behavior is a behavioral need of small ruminants. Thus, preventing such behavior would have negative implications for animal welfare.

Our results suggest that sheep and goats prefer the single components of hay and grass silage to a mixed ration of these components. Explanations for this could be that the animals seek variable mixing ratios throughout the day and/or because the sensory stimuli of the single components are lost or significantly reduced through mixing. The present study raises the question of whether mixed-ration feeding is acceptable for sheep and goats from a welfare perspective, as a mixed ration was shown to be less preferred than unmixed single components. Mixed-ration feeding limits small ruminants' natural behavior of selective feeding.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Swiss Cantonal Veterinary Office Thurgau, Frauenfeld, Switzerland.

Author contributions

RB: planning and conducting the experiment, data collection, data management, statistical analyses and visualization, and writing. MS: statistical analysis and visualization and co-writing. FD-M: planning and organizing the chemical feed analyses, advising on ruminant nutrition, and co-writing. NK: project management, planning the experiment, and co-writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1017669/full#supplementary-material

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Perspective: Opportunities for advancing aquatic invertebrate welfare

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Welfare considerations and regulations for invertebrates have lagged behind those for vertebrates, despite invertebrates comprising more than 95% of earth's species. Humans interact with and use aquatic invertebrates for exhibition in zoos and aquaria, as pets, research subjects, and important food sources. Recent research has indicated that aquatic invertebrates, in particular cephalopod mollusks and decapod crustaceans, experience stress and may be able to feel pain. With this article, we present results of a survey on attitudes of aquatic animal health professionals toward aquatic invertebrate welfare and provide practical recommendations for advancing aquatic invertebrate welfare across four areas of opportunity: use of anesthesia, analgesia, and euthanasia; development of less invasive diagnostic and research sampling methods based on 3R principles; use of humane slaughter methods for aquatic invertebrates; and reducing impacts of invasive procedures in aquaculture and fisheries. We encourage consideration of these opportunities to achieve far-reaching improvements in aquatic invertebrate welfare.

KEYWORDS

anesthesia, animal welfare, euthanasia, humane slaughter, refinement

Introduction

Despite comprising >95% of the animal species on Earth (1), attention to invertebrate animal welfare has lagged behind those for vertebrate animals. Although the interest in ethics and anesthesia related to vertebrate animal welfare has been increasing since the mid-twentieth century (2, 3), invertebrates have been less in the focus of welfare research and regulations. Aquatic invertebrates are displayed in zoos and aquaria, kept as pets, used as research animals, and serve as food sources for humans and other animals. Efforts to provide high quality care, to improve public perception and trust, and to extend ethical responsibilities to all veterinary patients and research subjects have driven the need to be mindful of aquatic invertebrate welfare.

Complicating our ability to discuss aquatic invertebrate welfare is the variable complexity of aquatic invertebrate nervous systems—from sponges which lack true nervous tissues (4) to cephalopods with roughly half a billion neurons (5). Cephalopods and decapod crustaceans are considered "advanced invertebrates" and have been the focus of the majority of research regarding invertebrate welfare. Cephalopods (e.g., cuttlefish, nautilus, octopus, and squid) have arguably the most complex nervous system found in invertebrates (6) and have a large body of literature devoted to exploring their nociceptive capabilities and pain perception (7–11). Decapod crustaceans (e.g., prawn, crab, lobster, and crayfish) have also been the subject of similar studies on nociceptive capabilities and pain perception (12–14) as well as indicators of stress (15–18). Less information is available for other taxa. Given the evidence of pain perception and stress in aquatic invertebrates, welfare considerations provide opportunities for advancements. In addition to the opportunities discussed in this paper, welfare can be improved through further minimization of stressors and the provision of species-appropriate housing, diet, water quality, social structure, and choices within an enriched environment, where appropriate (19–22).

Legal protections for aquatic invertebrates vary by country and whether the animals are used for research or human consumption. Cephalopods in research are protected in the European Union by Directive 2010/63/EU (23); decapod crustaceans were recommended for inclusion in this legislation (24) but were ultimately not included. This legal protection does not extend to animals intended for human consumption. Cephalopods and decapod crustaceans are protected in Switzerland (25), Norway (26), and New Zealand (27). Octopus are protected in the UK (28), although a recent publication by the London School of Economics reported strong evidence of sentience in cephalopod mollusks and decapod crustaceans (29). In Canada, cephalopods and "some other higher invertebrates" are protected (30). In the United States, invertebrates are not included in the Animal Welfare Act (31) but may be included for oversight by certain Institutional Animal Care and Use Committees if requested by the funding agency.

Here we present and discuss a survey on attitudes of aquatic animal health professionals toward aquatic invertebrate welfare and then provide practical recommendations for advancing aquatic invertebrate welfare across four areas of opportunity.

Current attitudes toward aquatic invertebrate welfare among aquatic animal health professionals

In November 2019, a 10-question informal, anonymous survey was distributed electronically to three veterinary medicine-focused professional email listservs to determine the attitudes of aquatic animal health professionals toward the welfare of aquatic invertebrates. The majority of the 112 respondents identified as veterinarians (87%) while others identified as animal care staff, pathologists, researchers, veterinary technicians or assistants, or veterinary students. Sixty-seven of 111 (60%) thought that invertebrates can feel pain and 52 of 61 (85%) thought that cephalopod mollusks could feel pain. Only 49% had attempted pain control in invertebrates. Seventy-five of 112 (67%) indicated that they strongly consider the welfare of the invertebrates when performing treatments, procedures, or euthanasia. Respondents indicated that they euthanized aquatic invertebrates most frequently due to illness (95%), followed by population control (20%), cosmetic reasons (15%), research (5%), diagnostics (2%), feed for other animals (2%), age-related reasons (2%), and health surveillance (1%). The most common methods for euthanasia, either individually or in combination, included immersion in tricaine methanesulfonate, otherwise known as MS-222 (58%), magnesium salts (52%), physical methods (30%), freezing (20%), immersion in alcohol (18%), and/or Aqui-S/clove oil/eugenol (13%). Less common methods included sodium pentobarbital (5%), removal from water (4%), isoflurane (2%), formalin (2%), 2-phenoxyethanol (1%), lidocaine (1%), propofol (1%), and "shock" (1%) which was not further defined. Fifty of 92 (54%) identified that they used a two-step process. The results of this survey highlighted the need for development and implementation of evidence-based guidelines to improve the welfare of aquatic invertebrates in various settings and as appropriate. Future research on the topic could benefit from a formal survey with more participants to enable further statistical analyses.

Opportunity 1: Promote the use of anesthesia, analgesia, and euthanasia

Anesthesia, analgesia, and euthanasia can provide great improvements to aquatic invertebrate welfare when appropriately implemented. Anesthesia can be used to immobilize aquatic invertebrates for physical examination, sample collection and procedures, and to reduce stress and the potential for injury for both animal and handler. Commonly used anesthetic concentrations have been previously reported for a limited number of species (32–36). The selection of anesthetic should be based on a knowledge of species biology, mechanisms of action of the agent, clinical judgment and if possible, recent literature, though even published methods should be critically evaluated. While hypothermia, carbon dioxide, and calcium-free seawater have been utilized as anesthetics, these procedures likely induce physiologic derangements, and their use may raise welfare concerns. Anesthesia for aquatic invertebrates typically involves immersing the animal in a solution (such as magnesium salts or 1–10% ethanol) or providing flow of anesthetic solution across the animal. Care should be taken that the solution is at the same temperature, pH, and osmolality as the animal's life support system water and is aerated to prevent hypoxia, and that water quality is monitored during prolonged procedures. Invertebrates should be frequently monitored and adjustments to the concentration of anesthetic made to maintain an optimal anesthetic plane. While MS-222 is commonly used in aquatic animal medicine, it may not be the best anesthetic choice for some invertebrate taxa as high concentrations are required which may lead to substantial changes in water chemistry that potentially impact animal health (37, 38).

When performing invasive or potentially painful procedures, the use of analgesic medications should be considered. However, there is a lack of information on appropriate analgesic medications for aquatic invertebrates. The few published research studies have focused on the use of local anesthetics such as lidocaine, given the conservation of sodium channels across species (39, 40). Lidocaine injections appear to have analgesic properties in cephalopods due to blocked afferent nerve signals and the prevention of behavioral responses to noxious stimuli (8, 11). Topical benzocaine decreased behavioral responses of glass prawn (*Palaemon elegans*) to noxious stimuli, also indicating potential analgesia (41). While morphine has been frequently used in decapod crustacean research, the observed results appear to be from sedation rather than analgesic properties (42). More evidence-based analgesic options are needed for all taxa of aquatic invertebrates.

Euthanasia is used to describe the act of ending the life of an animal in a manner that minimizes or eliminates pain and distress. Slaughter on the other hand is the act of killing animals for human or another animal's consumption (43) and is discussed in Opportunity 3. A follow-up anonymous survey was distributed electronically in May 2022 to four professional email listservs, predominantly aquatic animal veterinarians, to determine current euthanasia techniques for aquatic invertebrates. Of the 36 respondents who had euthanized an aquatic invertebrate in the previous 2 years, 92% identified as clinical veterinarians. The results of the survey are reported in Table 1.

To be considered euthanasia, an animal should be quickly rendered non-responsive and the method should minimize stress, be reliable, reproducible, and irreversible (44). A two-step approach is recommended for the euthanasia of aquatic invertebrates by the American Veterinary Medical Association (43). The first step should render the animal non-responsive and can include immersion in anesthetics such as magnesium salts ($MgCl_2$ or $MgSO_4$), clove oil, eugenol, or ethanol (1-10%). Injections of potassium chloride in direct proximity to the ventral nerve chord or injectable anesthetics can be used in crustaceans (45, 46). The second step should be unsurvivable and include physical or chemical destruction of the brain or major ganglia. Acceptable options for the second step include immersion in 70% alcohol, 10% formalin, or physical methods such as pithing, freezing, boiling, or sharp dissection. Methods that are unacceptable as a first or solo step include removal from the water to die by desiccation and hypoxia, freezing, or immersion in caustic chemicals (such as tissue fixative or 70% ethanol) (43).

Opportunity 2: Development of less invasive sampling methods for research and diagnostic procedures

Research protocols and diagnostic procedures in aquatic invertebrates often involve terminally collected samples which may not be sustainable considering population declines in many invertebrate species. As of 2021, the International Union for Conservation of Nature (IUCN) lists 1,661 invertebrate species as critically endangered or endangered and an additional 1,326 species as threatened (47). Furthermore, lethal sampling may become unacceptable due to changing public attitudes and increasing animal welfare concerns by the scientific and animal health communities.

In animal research, scientists are obligated to use the 3Rs (replacement, reduction, and refinement) as a framework for the humane treatment of animals. The 3Rs were originally developed in 1959 by Russel and Burch (48) to improve laboratory animal welfare but are generally applicable to any situation where animals and humans interact. Replacement refers to replacing the use of animals; this can include the use of *in vitro* and *in silico* models. Reduction refers to the use of appropriate experimental design to appropriately power a study and optimize the number of animals used, as well as the data collected from each animal. Refinement refers to minimization of the pain, suffering, distress, and harm experienced by research animals (49).

We support application of the 3Rs principles across the animal kingdom. In various research settings and for diagnostic testing, lethal sampling techniques can be replaced with non-lethal procedures, including collection of hemolymph, coelomic fluid, or tissue biopsies. Current guidelines for blood collection in mammals limit removal to 10% of the total circulating blood volume (50), but very few analogous recommendations exist for invertebrates. Hemolymph and coelomic fluid removal should be limited to the minimum amount necessary, and perhaps no more than 10% of the circulating volume until safe guidelines can be established through research. Tissue biopsies should also be kept to the minimum practical size needed to fulfill sampling objectives. Only a few milligrams of tissue are necessary for conservation genetics and other molecular testing. Non-lethal sampling has been performed in sponges, corals, crustaceans, insects, echinoderms, and mollusks (51). Examples of non-lethal procedures include *in vivo* solid phase microextraction using a fiber inserted near the mouth of the

TABLE 1 Responses from an electronically delivered survey on currently used methods for euthanasia of aquatic invertebrates.

	Solo step method	Taxa					
		Bivalve and gastropod mollusks	Cephalopod mollusks	Cnidarians	Crustaceans	Echinoderms	Horseshoe crabs
	Number of respondents (n)	12	23	24	27	21	13
	Single/solo step performed	5 (41.7%)	4 (17.4%)	9 (37.5%)	8 (29.6%)	6 (28.6%)	4 (30.8%)
Immersion	MS-222	2 (16.7%)	1 (4.3%)	1 (4.2%)	3 (11.1%)	1 (4.8%)	0
	Clove oil/Eugenol/AquiS®	0	1 (4.3%)	1 (4.2%)	2 (7.4%)	0	0
	Magnesium chloride or magnesium sulfate	2 (16.7%)	2 (8.7%)	5 (20.8%)	2 (7.4%)	4 (19.0%)	0
	1–10% Ethanol	0	0	0	1 (3.7%)	1 (4.8%)	0
	>50% Ethanol	0	0	0	2 (7.4%)	0	0
	Formalin	0	0	0	0	0	0
	2-PE	0	0	0	0	0	0
	KCl	0	0	0	0	0	0
Injection	Pentobarbital	0	0	0	1 (3.7%)	0	4 (30.8%)
	Lidocaine	0	0	0	0	0	0
	Propofol	0	0	0	0	0	0
	Physical method	0	0	1 (4.2%)	0	0	0
	Freezing	1 (8.3%)	1 (4.3%)	1 (4.2%)	1 (3.7%)	0	0
	Removal from water	0	0	0	0	0	0
	Other	0	0	0	1 (3.7%)	0	0

	1st step method	Taxa					
		Bivalve and gastropod mollusks	Cephalopod mollusks	Cnidarians	Crustaceans	Echinoderms	Horseshoe crabs
Immersion	MS-222	0	1 (4.3%)	5 (20.8%)	4 (14.8%)	3 (14.3%)	1 (7.7%)
	Clove oil/Eugenol/AquiS®	0	1 (4.3%)	1 (4.2%)	5 (18.5%)	2 (9.5%)	2 (15.4%)
	Magnesium chloride or magnesium sulfate	6 (50%)	10 (43.5%)	9 (37.5%)	7 (25.9%)	10(47.6%)	3 (23.1%)
	1–10% Ethanol	3 (25%)	7 (30.4%)	4 (16.7%)	2 (7.4%)	3 (14.3%)	0
	>50% Ethanol	1 (8.3%)	5 (21.7%)	2 (8.3%)	0	2 (9.5%)	0
	Formalin	0	0	1 (4.2%)	0	0	0
	2-PE	0	0	0	1 (3.7%)	1 (4.8%)	3 (23.1%)
	KCl	0	0	0	3 (11.1%)	0	1 (7.7%)
Injection	Pentobarbital	0	1 (4.3%)	0	2 (7.4%)	1 (4.8%)	1 (7.7%)
	Lidocaine	0	0	0	0	0	0
	Propofol	0	0	0	0	0	0
	Physical method	0	0	0	1 (3.7%)	0	0
	Freezing	0	0	2 (8.3%)	0	0	0
	Removal from Water	0	0	0	0	0	1 (7.7%)
	Other	0	0	0	0	0	0

(Continued)

TABLE 1 (Continued)

	2nd step method	Taxa					
		Bivalve and gastropod mollusks	Cephalopod mollusks	Cnidarians	Crustaceans	Echinoderms	Horseshoe crabs
	MS-222	0	0	0	0	0	0
	Clove oil/Eugenol/AquiS	0	0	0	0	0	0
	Magnesium chloride or magnesium sulfate	0	3 (13.0%)	2 (8.3%)	0	0	0
Immersion	1–10% ethanol	0	1 (4.3%)	0	0	1 (4.8%)	0
	>50% ethanol	0	0	3 (12.5%)	2 (7.4%)	0	1 (7.7%)
	Formalin	1 (8.3%)	0	5 (20.8%)	2 (7.4%)	2 (9.5%)	1 (7.7%)
	2-PE	0	0	0	0/27	0	0
	KCl	0	2 (8.7%)	0	4 (14.8%)	0	2 (15.4%)
	Pentobarbital	0	4 (17.4%)	0	4 (14.8%)	0	4 (30.8%)
	Lidocaine	0	0	0	1 (3.7%)	0	0
	Propofol	0	0	0	0	0	0
Injection	Physical method	1 (8.3%)	11 (47.8%)	1 (4.2%)	7 (25.9%)	5 (23.8%)	2 (15.4%)
	Freezing	7 (58.3%)	4 (17.4%)	11 (45.8%)	10 (37.0%)	11 (52.4%)	2 (15.4%)
	Removal from water	1 (8.3%)	0	1 (4.2%)	1 (3.7%)	1 (4.8%)	0
	Other	0	0	0	1 (3.7%)	0	0

Respondents were permitted to select all options that apply so the total count of percentages are >100%.

animal to evaluate plastic contaminants in corals and bivalves (52) and the use of dragonfly fecal pellets and shed exoskeletons for DNA extraction (53). Further refinement can include the use of anesthetics and analgesics for invasive procedures. Handling techniques can be evaluated and improved to minimize stress and harm. If non-lethal sampling is performed but animals must be permanently removed from the wild, a plan to provide life-long care presents an opportunity for placement in educational or display settings. For example, if planned in advance, disposition to public aquaria may be an option for some non-releasable invertebrates, depending on capacity and institutional collection plans.

In cases where invasive sampling cannot be avoided, sharing samples with other researchers can reduce the need for additional specimen collection (54). If lethal sampling is required, aquatic invertebrates should be euthanized prior to sampling. Due to concerns over sample quality, invertebrates are often terminally sampled without methods rendering them non-responsive prior to sampling. However, several studies have demonstrated that high quality samples can still be obtained from euthanized animals. High quality RNA was successfully extracted from sea stars immersed in $MgCl_2$ prior to sampling (55) and jellyfish immersed in $MgCl_2$ provided useful samples for NMR-based metabolomics (56).

Opportunity 3: Use of humane slaughter methods for aquatic invertebrates

Aquatic invertebrates including non-cephalopod mollusks (e.g., bivalves and gastropods), crustaceans, cephalopod mollusks, jellyfish, sea cucumbers, and sea urchins are commonly consumed by humans with 41 million tons captured or cultured in 2018 (57). While euthanasia methods are published for many of these taxa, there is a lack of peer-reviewed literature evaluating humane stunning and slaughter methods. The only taxon with published information on humane slaughter are decapod crustaceans (58). Surprisingly, while cephalopods are the focus of much research on sentience and pain perception, no published article could be found on appropriate slaughter techniques for this taxon, as of April 2022. Methods for cephalopod slaughter include decapitation without prior stunning (59), "clubbing, slicing the brain, reversing the mantle, and asphyxiation in a suspended net bag", none of which are considered to be humane (29).

The debate on humane slaughter methods for decapod crustaceans started in the 1950s with publications by Baker and Gunter (60, 61). There is contradictory evidence on whether slowly warming live animals or placing live animals in boiling water is humane (62, 63), but boiling lobsters alive has been banned in New Zealand (64), Norway (26), Switzerland (65), and certain parts of Italy (66). Ice slurries and electrical shock may paralyze crustaceans, but neural circuits still remain intact and functional so these methods are likely best used after rendering the animal insensible (67). Based on the available scientific evidence, all animals should have their nerve ganglia destroyed prior to cooking to prevent any potential suffering (60, 68). A commercially available stunning device, the CrustaStun (Mitchell & Cooper, Uckfield, England) that is recommended by the Royal Society for the Prevention of Cruelty to Animals (RSCPA), can be used to stun crustaceans prior to boiling and has been shown to arrest nervous activity after use (69).

Regardless of the method for slaughter chosen, stress should be minimized throughout the supply chain and animals should be killed quickly to avoid unnecessary suffering and pain. More evidence-based and species-appropriate methods are needed for practical humane slaughter of aquatic invertebrates, particularly for cephalopod mollusks.

Opportunity 4: Reduce the impact of invasive procedures in aquaculture and fisheries

Crustaceans have been shown to experience stress and likely have the capacity to feel pain, which should be considered during processes from collection to slaughter. Industry practices that might be adjusted to minimize stress include decreasing the trawling duration, live transport duration, and handling needs (70). Additional good practice recommendations include maintenance of the animals' thermal preference zone, provision of good water quality, and allowance of recovery periods (71). Anesthetics can also be used to decrease stress throughout the supply chain from collection to slaughter. Isobutanol, a food safe anesthetic, reduced ammonia concentrations and mortalities during live transport of tropical spiny lobsters (*Panulirus* spp.) (72).

Crabs in fisheries worldwide have their claws manually removed followed by release back into the water. Live declawing is performed with brown crabs (*Cancer pagurus*) (73), stone crabs (*Menippe* spp.) (74), and fiddler crabs (*Uca tangeri*) (75). While crustaceans do autotomize claws naturally, manual declawing is more stressful and causes significantly higher mortality than natural autotomy (73). This practice is often considered better than whole crab landing, based on the assumption that these animals survive and regenerate their claws, while remaining in the fishery. However, mortality was >60% in stone crabs with both claws removed (76) and regenerated claws only comprised 3% of legal stone crab landings (77), indicating that this practice is little or no more sustainable than whole crab harvest. Crabs that survive declawing show decreased feeding (74, 78) and decreased reproductive fitness (75, 79). Based on animal welfare concerns and negligible population benefits, declawing may not be preferable over humane harvest and slaughter.

Brown crabs are often transported alive, and mechanisms are needed to prevent them from damaging each other during transport. In the Irish fishery, they undergo a process known as nicking, which involves cutting the ligament under the dactylus of the claw since their claw shape makes traditional banding used in other crustaceans challenging (80). Nicking results in hemolymph loss, risk of infection, inability to molt, and increased mortality (81–83). Nicked crabs had higher hemolymph glucose, lactate, and refractive index, indicating they experienced increased stress (80). In the Norwegian fishery, the crabs are not nicked and are instead transported dry (84). However, emersion can also result in welfare issues, particularly at higher temperatures (85). Adapted banding techniques [e.g., Elastrator (castrator) rings combined with a wooden dowel through the claw] could be considered (86). Finding a solution that balances crab welfare with the needs of the fisheries offers an opportunity for research.

In shrimp aquaculture, eyestalk ablation is performed to induce female broodstock to spawn, since the eyestalks are a source of vitellogenesis-inhibiting hormone (VIH) which is a negative regulator of crustacean reproduction (87). Following eyestalk ablation, shrimp exhibit stress-related behaviors including erratic and spiral swimming, rubbing, and tail flicking, which are prevented by topical anesthetic application (88, 89). Beyond the stress and potential pain caused by handling and the procedure, eyestalk ablation can also impact the immune system of shrimp (90). Non-ablated broodstock females appear to perform at a similar level as ablated females, with larvae that are more resilient to typical pathogens and environmental stress (91, 92). As eyestalk ablation carries negative health and welfare consequences, evaluation of alternatives could be beneficial. Switching from a 1:1 ratio of females to males to a 1:2 ratio improves performance without ablation (91) and a single injection of anti-GIH monoclonal antibody was shown to have similar performance to eyestalk ablation (93).

Conclusions

Aquatic animal health professionals believe that aquatic invertebrates, especially cephalopods, can feel pain. However, <50% have used analgesia during invasive procedures with aquatic invertebrates, likely due to a dearth of well described effective options. This highlights the need for more research on appropriate anesthetic and analgesic options for aquatic invertebrates. While the discussion of pain perception in invertebrates is important, the ability to feel pain is not a prerequisite for promoting positive animal welfare in aquatic invertebrates. Many cost- and time-effective opportunities for the improvement of aquatic invertebrate welfare exist and can be appropriate in various settings. We advocate the use of these advancements and further investigations in this underrepresented but important field of animal welfare.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SW and NS conceptualized the presented idea. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author AN is employed by ZooQuatic Laboratory, LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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