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Management of dairy heifers: can operant conditioning be an effective and feasible tool to decrease stress and ease animals' close contact and handling?

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ABSTRACT

Beside health monitoring, a regular check of dairy heifers' growth rate is desirable, but it is rarely done since procedures that require restraint and handling can be associated with substantial stress for both animals and farmers. Inexperienced heifers, especially if they are highly responsive to humans, may find restraint and handling potentially aversive. This study investigated whether training heifers of different age and responsiveness toward humans (RTH), through operant conditioning, could reduce stress in animals, ease close contact and handling, and be feasible in terms of farmer's effort. We assessed 60 Holstein heifers of 2 age classes (Young, n = 29, 291 ± 39; Old, n = 31, 346 ± 62 d) according to the Avoidance Distance Test (ADT) and classified them as Confident (C, n = 20), Neutral, (N, n = 21) or Non-confident (NC, n = 19). Half of the heifers of each age and RTH class was trained (Tr, n = 29), whereas the other half was not (NTr, n = 31). The Tr heifers were subjected to target training for 8 sessions and positively reinforced with feed to allow being touched on the muzzle, rump and perineum. In case a heifer refused positive reinforcement, the trainer stepped back as negative reinforcement. In the last week of the experiment the effect of training on the reaction to handling was assessed in all heifers. We measured heart rate (HR), root mean square of successive inter-beat interval differences (RMSSD) and fecal cortisol metabolites (FCMs). The presence of behavioral distress signs was recorded as well. The ADT was performed a second time 24 h after the measuring session. All the trained heifers, regardless of RTH class, successfully accomplished the target training task in 6 sessions, spending on average 25.3 s per session, each. All the trained heifers allowed to be touched on the rump and perineum at the end of the 4th session. Training NC heifers required more time compared with the others. Trained heifers showed higher RMSSD (14.2 vs. 16.9), indicating a lower vagal tone, and thus, a slightly lower stress level than NTR. Training did not lead to differences in HR, FCMs or presence of stress behavioral signs. NC heifers had the highest mean baseline FCM values (38.4 vs. 30.3 vs. 29.1 ng/g) compared with N and C. NC heifers showed also the lowest value of FCMs, 12 h after the measuring session (36.7 vs. 44.6 vs. 49.7 ng/g), likely due to a decreased responsiveness of the adrenal gland to a stressor. The average avoidance distance decreased between the beginning and the end of the experiment, especially for N and NC heifers, regardless whether they were trained or not. These results show how using operant conditioning on some heifers, not only decreased their vagal tone, but reduced the responsiveness to humans of all the animals, trained and not trained; in the latter case, through non-associative learning, such as habituation.

Key words: cattle training, negative reinforcement, responsiveness to humans, heart rate variability, fecal cortisol metabolites

INTRODUCTION

Dairy heifers are an integral part of any dairy cow farm and account for more than 12% of total expenses, with the feed accounting for more than 60% of the whole cost (Gabler et al., 2000). It is widely recognized that management and care of dairy heifers directly affect productivity during their first and subsequent lactations (Zanton and Heinrichs, 2016). However, in current farms, beside heifers' health, a consistent monitoring of other important parameters, such as growth rate and feed efficiency, is not always carried out. Possible reasonable causes of this include the farmers' lack of time, the underestimation of the importance for heifer breeding, and last but not least, the difficulty in physically handling them (Bertenshaw et al., 2008). Heifers are often not used to handling, and therefore, when stressed can cause injuries to the farm personnel

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(Bertenshaw et al., 2008). Additional factors that make it more difficult to physically handle heifers include: i) the lack of restraining facilities such as headlocks, and ii) suboptimal conditions, which contribute to increase animals' stress, such as overcrowding, dirty stables and unbalanced diets. Keeping the heifers' healthy and consistently monitoring their growth is a key factor to reach their potential milk yield as cows (Svensson and Hultgren, 2008).

Gentle and pleasant tactile contact has been proven beneficial to human–animal interactions (Westerath et al., 2014; Waiblinger et al., 2004) and positive handling practices contribute to reduce animals' fear (Breuer et al., 2000; Hemsworth et al., 2000; Chen et al., 2016) and might facilitate routine operations such as fecal sample collection, artificial insemination, pregnancy diagnosis and, diseases diagnosis and treatment. A way to reduce fear toward humans is to let them change their behavior on the basis of their experience; in other words, they have to learn (Mellen and Ellis, 1996), through different techniques, to cope with the presence of farmers and to be handled by them. Helping heifers learn to be handled can therefore become a valid tool to facilitate routine management operations. Calves, who were offered a suitable amount of milk during injections, showed to spontaneously accept the injections (Ede et al., 2018), and sheep that were offered barley during shearing took less time to return to the shearing place than control sheep (Rushen, 1996).

Learning how to cope with humans might also reduce the stress due to the interactions between animals and farm personnel, lower the frequency of animals' difficult behaviors, the risk of accidents at work, as well as ease the measurement of performance (Bertenshaw et al., 2008). Among the pros of improving human-animal relationship, some authors also reported that cows showing signs of nervousness have a lower milk yield than calm cows (Breuer et al., 2000; Hedlund and Løvlie, 2015). Target training is a technique used very often in zoos to approach the animals, drive them around or outside their enclosures, and shape their behaviors to accomplish specific tasks, such as undergoing specific diagnostic procedures or therapies (Dadone et al., 2016). With regard to domestic animals, target training is also used to load horses in their trailers, avoiding aversive procedures (Carrol et al., 2022; Ferguson and Rosales-Ruiz, 2001) and in dairy cattle to study their pre-calving isolation behavior (Rørvang et al., 2018) and anticipatory and play behavior (Heinsius et al., 2023).

The application of learning techniques in cattle, however, has not always given consistent results. For example, with regard to the adaptation to milking of cows upon first parturition, the effectiveness of training techniques seems to vary according to the animal′s temperament and responsiveness to humans (**RTH** – for abbreviations see Table S1, https://doi.org/10.5281/zenodo.10474723, Marchesini et al., 2024), and the training technique itself (Bertenshaw et al., 2008; Sutherland et al., 2012, Kutzer et al., 2015). Among the available training techniques, habituation is a non-associative learning process, in which the frequency of an existing behavior is reduced in response to a stimulus which is repeatedly presented (Levitan and Kaczmarek, 1991; Dirksen et al., 2020a). With regard to associative training techniques, classical conditioning is based on the association of an originally neutral stimulus, which does not cause a response from the animal, with a stimulus that instead causes a reaction from the animal; this will ensure that, once the animal has been properly conditioned, the initially neutral stimulus will provoke the response from the animal, even without the second stimulus being present (Rescorla and Wagner, 1972; Mellen and Ellis, 1996; Lomb et al., 2021). Operant conditioning consists of associating an animal′s behavior with a response from an operator, which can have a positive or negative valence to the animal (Rescorla and Wagner, 1972; Mellen and Ellis, 1996; Lomb et al., 2021). In case of a positive valence (reinforcement), the behavior will be more likely to occur in the future, on the contrary, if the response has a negative valence (punishment), the behavior will occur with lower probability (Mellen and Ellis, 1996). Reinforcement can be both positive and negative: it is positive when something pleasant is added, and negative, when something unpleasant is taken away from the animal (Mellen and Ellis, 1996). Reinforcement can be used with the aim of counterconditioning animals toward stimuli that are initially perceived as aversive (Joyce-Zuniga et al., 2016). Operant conditioning is often used with zoo animals or marine mammals to teach them to perform certain gestures to simplify their management by the keeper (Behringer et al., 2014; Dadone et al., 2016) and it is also defined as husbandry training. Operant conditioning usually requires a person to work individually with an animal, and can be extremely time consuming (Dadone, et al., 2016). Lomb et al. (2021), for example, found that operant conditioning was more effective than habituation to reduce the aversiveness of an injection in heifers, but the training took up to 85 sessions, which represents quite an effort in terms of time for a farmer. However, operant conditioning, if properly planned and adapted to be used with animals in group, might also become a reality applicable to the dairy cow sector with satisfactory results.

The aim of this study was to investigate whether the application of operant conditioning to a group of dairy heifers of different age and RTH, is effective in reducing stress, easing the close contact and handling of the heifers by humans, and feasible in terms of farmer's effort.

MATERIALS AND METHODS

Ethical Statement

Experimental procedures were carried out in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the animal welfare committee (Organismo Preposto al Benessere Animale committee – OPBA – protocol number 16206 (02–02–2021) of Padova University. Furthermore, this study complies with the ARRIVE guidelines (Kilkenny et al., 2010).

Location, heifers, feeding and experimental design

The experiment took place from February to April 2021 at a commercial dairy farm, located in the Vicenza province (Veneto region, Northeast Italy). The farm reared 230 loose housed cows in milk, with 40% being primiparous. The farm was characterized by an average daily milk yield of 33 L/cow and an average parity of 2.1 lactations. The study involved a group of Holstein heifers (n = 60) allocated to 2 different pens based on age: Young (n = 29; 291 ± 39 d) and Old (n = 31, 346 ± 62 d). They were raised in the same barn, in loose housing conditions, in 2 different pens with concrete-slatted floors. Both pens had 40 cubicles bedded with mattress and straw, and 24 headlocks at the feed bunk, each. Heifers were fed a total mixed ration once a day around 7 a.m. and fresh water was always available in troughs. The TMR was the same for both pens and was mainly based on wheat, corn and sorghum silages and a nucleus made of soybean meal, vitamins and minerals, as reported in Table 1.

All the 60 heifers were classified in 3 RTH classes, based on the avoidance distance test (**ADT**), as reported by Kutzer et al. (2015): Confident (**C**, n = 20), Neutral (**N**, n = 21) and Non-confident (**NC**, n = 19). About half of the heifers of each RTH class were subjected to an operant conditioning treatment (**Tr**, n = 31) while the rest were considered as a control (**NTr**, n = 29). Tr and NTr heifers had similar average ADT values and standard deviation (SD). Details on animal distribution regarding age, RTH class and training are reported in Table 2.

As described below and reported in Figure 1, the experiment lasted 12 weeks, Tr heifers were trained for 20 sessions using operant conditioning to be approached and handled by a person. At the end of the experiment all heifers were re-tested for the ADT. Multiple measures and samples were collected at the beginning, during, and at the end of the experiment, as reported in Figure 1.

Table 1. Ingredients and composition of the total mixed ration (TMR)

Item	(% of DM unless otherwise indicated)
Ingredients	
Wheat silage	48.0
Sorghum silage	25.7
Nucleus[1]	16.5
Corn silage	9.8
Chemical composition (g/kg) [2]	
CP	120
NDF	457
ADF	247
Starch	152

[1]1 kg of nucleus corresponds to: 0.027 kg of mineral mix, 0.038 kg of bicarbonate, 0.016 kg of vitamins, 0.92 kg of soybean meal.

[2]DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Measures and sampling at the beginning of the experiment, before conditioning

On the first day of experiment all heifers were tested twice for RTH using an adaptation of Kutzer's version of ADT (Kutzer et al., 2015). The cows were approached by experienced unknown personnel when they were standing at the feed bunk, at a speed of one step per second starting from a distance of about 4 m. The ADT was applied about half an hour before the usual TMR delivery time with closed headlocks. When the heifers tried to move away, the person stopped and the distance from the stretched hand of the operator to the muzzle was recorded using a laser meter (BOSCH DLE 50, Robert Bosch S.p.A., Gunzenhausen-Schlungenhof, Germany; range: 0–30 m; precision ± 1.5 mm). The ADT was replicated immediately upon the first measure was collected from all the heifers, and the 2 values were averaged for each heifer. In case a heifer had accepted to be touched on the muzzle, but immediately after tried to escape, the distance was recorded as 0.05 m. Otherwise, when a heifer allowed to be touched on the muzzle for at least 5 s, the distance was recorded as 0 m (Kutzer et al., 2015). The heifers were classified

Table 2. Number of heifers distributed by age, treatment and responsiveness to human (RTH) class

	Tr [1]			NTr			
	C [2]	N	NC	C	N	NC	Total
Old	4	6	6	5	5	5	31
Young	6	5	4	5	5	4	29
Total	10	11	10	10	10	9	60

[1]Tr = trained; NTr = not trained;.

[2]C = confident; N. neutral; NC = Non-confident.

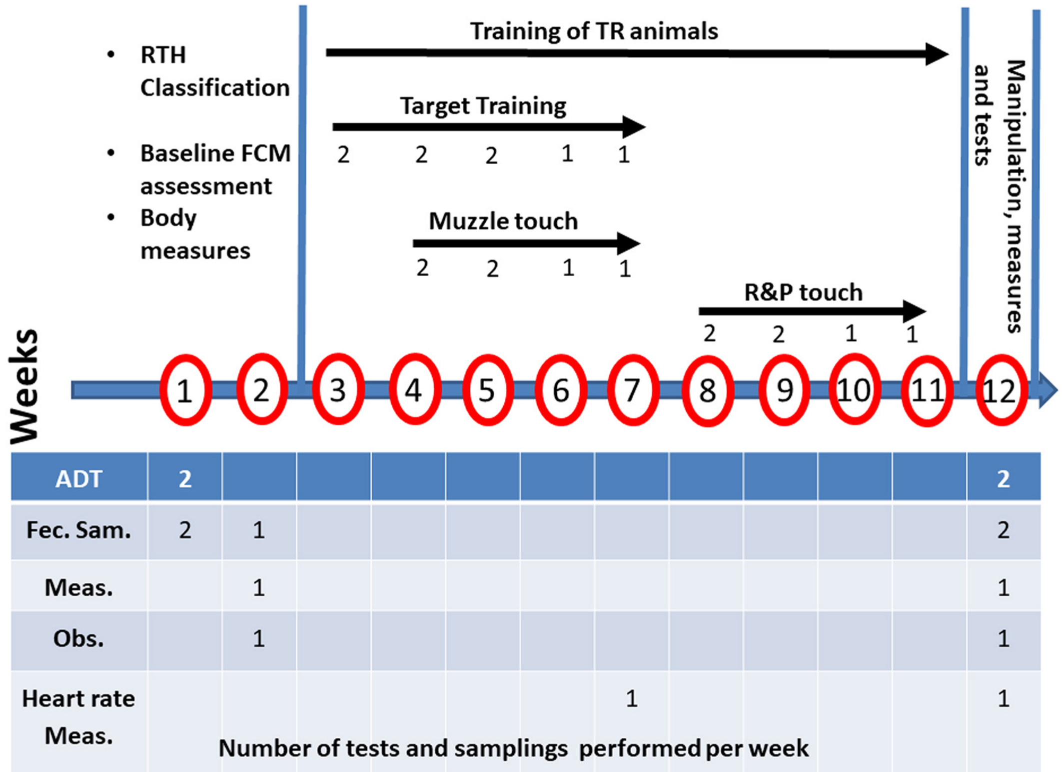

Figure 1. Experiment's activity schedule along a 12-week period ADT = avoidance distance test; Fec. Sam = fecal samples; FCM = fecal cortisol metabolites; Heart rate Meas. = heart rate measures; Meas = measures of heart girth; Obs = behavioral observations of tail clamped between hind legs, lowered head, lowered ears, eyes wide opened, stepping and kicking; R&P = rump and perineum; RTH = reactivity to humans.

in 3 RTH groups of the same size according to tertiles of the ADT outcomes: Confident (n = 20, ADT $\leq$0.45 m), Neutral (n = 21, 0.45 > ADT $\leq$1.05 m) or non-confident (n = 19, ADT >1.05 m). For each heifer, feces were then collected every other day, for 3 times, approximately 4 h after feed distribution. Fecal cortisol metabolites (**FCMs**) were analyzed to evaluate baseline adrenocortical activity for each animal (Palme, 2019). Fresh fecal samples were collected from the ground immediately after deposition, or if not possible, directly from the rectal ampulla. All samples were immediately frozen and stored at −20°C until analysis.

Heart girth was measured on all heifers, to calculate the animal's weight using the formula suggested by Heinrichs (1992).

$$Y = b0 + b1X + b2X^2 + b3X^3$$

Where, Y = body weight; b0 = the intercept; X = the heart girth; bl, b2, and b3 = regression coefficients.

During these measurements, behavioral observations were made, as reported by Kutzer et al. (2015): While an operator was measuring the heart girth of each heifer using a tape meter, 2 experienced observers, blind to the heifer's treatment or RTH class, either behind or in front of the heifer, made the behavioral observations to record the outcomes, and the time spent for each animal on a tablet (iPAD MYMH2TY/A, Apple Inc., Cupertino, US). At the same time, behaviors were video recorded using a hand-held camera to have the possibility of double checking the results. The observer behind the animal detected and recorded the number of steps and kicks performed during measuring, and the presence or absence of curved back and tail clamped between hind legs. Stepping is defined as weight displacement with the foot elevated less than 15 cm off of the ground, whereas kicking is characterized by the hoof lifted at least 15 cm (Kutzer et al., 2015). The observation detected by the observer in front of the heifers included the presence or absence of ears flat on the head, lowered head and wide opened eyes (Kutzer et al., 2015).

Conditioning procedures

As reported in Figure 1, 20 sessions of conditioning with each Tr heifer were performed in 9 weeks: 8 sessions of target training (TT), 6 sessions of positive reinforcement to condition heifers to let be touched on the muzzle (MT) and 6 sessions to let be touched on the rump and perineum and gently grabbed at the tail (**R&P**). The MT sessions were performed upon TT on the same days, whereas the R&P sessions were performed on different days from the 8th week on, as reported in Figure 1. Training sessions were performed at the feed bunk, upon the distribution of TMR, to ease the approach of the heifers, by 2 skilled trainers, who alternated periodically. Trainers tested and defined together the training procedures and methods to be used on some dairy cows, before the beginning of the experiment. Trainers knew the overall aim of the project, but they were not aware of the RTH class of the heifers to be trained. For TT, MT, and R&P, initially, 2 sessions per week (on Tuesday and Friday) were conducted during wk 3–5, 4–5, and 8–9, respectively. Subsequently, as the heifers demonstrated ease in performing the required tasks, the frequency was reduced to one session per week for wk 6–7, 6–7 and 10–11, respectively (Figure 1).

In this case, target training was chosen because it exploits animal curiosity toward new objects, and allows to approach the heifers without the need of direct contact. The target, a stick with a tennis ball fixed at the far end, was brought close (from 30 to 60 cm) to the animal's muzzle by the trainer, and the heifer was rewarded by the trainer's gloved hand every time it touched the target; The word "*brava*," said by the trainer, was used to shape the heifer's touching the target with her nose and paired with the reinforcement presentation (Ferguson and Rosales-Ruiz, 2001).

The goal was for the heifer to touch the target 3 times within 2 min and the time necessary to reach the goal was recorded. When the task was not accomplished, the number of touches obtained within 2 min was recorded. Positive reinforcement was initially tried for all Tr heifers, with the reward consisting of half a handful of pellets for calves or dry cows' ration according to their preference. For those heifers (n = 11) who were uncomfortable around humans, upon their refusal of the feed reward, a negative reinforcement was applied. The latter consisted in removing the unwanted presence of the operator by taking a step away and diverting the gaze from the animal, looking down and aside (Wergård et al., 2015; Fernandez, 2020). Negative reinforcement was then replaced by positive reinforcement once the heifer began accepting the feed reward. The first 3 sessions of training were done with the heifers closed in the headlocks, to ease the approach to all the heifers. Then, in the following sessions, when the heifers got habituated to the procedure, the headlocks were left open, leaving the heifers free of stepping away at any time. Since the number of heifers exceeded the number of headlocks, each session lasted long enough to allow all the heifers to come to the feed bunk. From the third session onward and for the following 6 sessions, in addition to target training, MT was performed (Figure 1). In MT the trainer tried to touch the heifers' muzzle with a hand, to see which of them allowed to be willingly touched and which did not. MT was trained since touching the head could be useful for different reasons, such as checking the ear tag number in case it is covered by hairs, sampling mucus or saliva, placing a ear tag or a collar, etc. When heifers accepted to be touched, we used patting as positive reward, since we took for granted that the heifer appreciated the contact. On the contrary, if an heifer refused to be touched we retreated. During the last 4 sessions, the time that each heifer allowed to be touched on the rump and perineum, and be grabbed at the tail (**R&P**), by a second operator, meanwhile the first was performing TT, was also recorded. This handling simulated the basic procedures needed for rectal palpation and blood sampling from the tail. When the trainer began TT, the second operator approached the animal following a standardised procedure, getting closed to the animals from behind, walking very slowly and speaking softly. The trainer first gently touched the rump, with the left hand, then gently grabbed the tail with the right hand. The duration from when the trainer grabbed the tail and touched the rump to the heifer retreated was measured. The task was considered fully accomplished after 15 s, that, empirically, was reckoned to be the average time needed to collect blood from the tail or feces from the rectal ampulla. TT was performed to distract the heifer for the time necessary to let the second person approach and apply the R&P for 15 s.

Procedures performed during the conditioning period

For each animal, heart rate (**HR**) per minute and one measure of heart rate variability, the Root Mean Square of Successive inter-beat interval Differences (**RMSSD**), which indicates the vagal activity (Kutzer et al., 2015), were measured. RMSSD and HR were performed twice and averaged, at wk 6, (2 following days), after 10 training sessions, with heifers in standard conditions and without being trained or disturbed, and a second time, at wk 12th (single recording), during the final measuring session. The 2 measures represented the basal condition during the experiment, and the condition during handling, in both Tr and NTr heifers. Heart rate and RR intervals were collected using the Polar Equine Belt (Polar Electro Oy, Kempele, Finland) fitted with the heart rate Polar H10 sensor and Polar Equine App (Polar Electro Oy, Kempele, Finland). The heifers were equipped with the chest belt approximately one hour before the recording to make the heifers adapt to the belt (Kutzer et al., 2015; Wierig et al., 2018). The belt was applied at the heart girth, with the 2 electrodes on the left side of the body, without shearing the heifers, but applying a thick layer of ultrasound gel between the electrodes and the fur. The recording of HR and RMSSD were done in the morning upon TMR distribution, and lasted 6 min for each heifer (Kutzer et al., 2015; Sutherland et al., 2012). To calculate the RMSSD, the length of RR intervals, which is defined as the distance between 2 consecutive R peaks (ms), was measured. Consecutive RR intervals that differed by more than 100 ms were considered as outliers and removed. RR values lower than 350 ms and higher than 1050 ms, were removed as well, as suggested by Wierig et al. (2018), since they were not physiologically possible and therefore considered measurement errors. The RMSSD for each animal was calculated through the formula suggested by Wierig et al. (2018):

$$RMSSD = \sqrt{\frac{1}{N-1}\left[\sum_{j-1}^{N-1}\left(RR_{j+1} - RR_{j}\right)^{2}\right]},$$

where, RMSSD is the root mean square of successive inter-beat interval differences and N is the number of the RR interval terms.

Procedures performed during or immediately after the handling and measures performed at the end of the experiment

In the last week of the experiment (wk 12th, Figure 1), all the heifers were subjected to handling, as mild stressor, to verify the effect of training on the stress response. Handling included, the measurement of heart girth, also used to estimate the body weight, and rectal palpation. HR and RMSSD during handling were measured and calculated, respectively, and behavioral observations were done during the whole procedure. In the evening of those same days, about 12 h afterward, feces samples were collected to assess FCMs reflecting the acute stressor (Palme, 2019). At the end of the experiment, the ADT was measured twice by an unknown observer through the same procedure described above, to see whether its value changed or not from the beginning.

Analytical methods

The TMR and feces nutrient composition was determined through the following procedures: #934.01 for DM, #2001.11 for CP, #996.11 for starch as described by AOAC (2005) and ANKOM Technology (2008) for NDF (with amylase and sodium sulphite), ADF and ADL. With regard to FCM extraction, feces samples were immediately frozen after the collection. Feces (0.5 g) were suspended in 5 mL of methanol (80%), centrifuged and then an aliquot of the supernatant was diluted with assay buffer and eventually transferred into an 11-oxoetiocholanolone enzyme immunoassay, measuring 11,17-dioxoandrostanes. The EIA has been described in detail by Palme and Möstl (1997) and has been successfully validated for use in cattle (Palme et al., 1999).

Statistical analysis

All data were analyzed using SAS software (2012, release 9.4; SAS Institute Inc., Cary, NC). Data on the time spent by the heifers to perform target training (8 sessions), after being normalized through natural logarithm, were analyzed by an ANOVA mixed model using the heifer ID as random and repeated effect and age (9 or 12 mo), RTH (C, N or NC), training session (8 levels) and their interactions as fixed effects. The estimates were reported as back transformed data using an exponential function. The same model was also used for RT (4 sessions). The analysis on the use of negative reinforcement during TT and the ease of being touched by operators during MT were done by comparing K proportions test with the Marascuilo procedure for pairwise comparisons. Body weight, ADT, HR and RMSSD and FCM were analyzed by an ANOVA mixed model using heifer as random and repeated effect and age (9 or 12 mo), RTH (C, N or NC), training (Tr or nTr), period (**P1**,before the final handling and **P2**, during or after final handling) and their interactions, as fixed effects. With regard to behavioral stress response, all data concerning tail clamped between hind legs, lowered head, lowered ears, eyes wide opened, stepping and kicking, were considered as binary variables (absent "0" or present "1"). Although stepping and kicking were recorded as number of steps and kicks performed per minute, since many heifers did not show any steps or kicks, and in the others, the number of kicks and steps was low, it was decided to consider those variables as binary as well, assigning "0" to the lack of kicks and steps and "1" when at least one kick or step was present. A generalized linear model with binomial distribution and logit link function was used to estimate the risk of performing each behavior as a function of age (9 or 12 mo), RTH (confident, neutral or non-confident) and training. The values of interactions among factors were reported in tables only when significant.

RESULTS

All the heifers were successfully trained to accomplish the task of touching the target 3 times within 2 min. With regard to the average time spent to accomplish that task (Figure 2), 5 training sessions were enough to significantly reduce the training time from 40 to 19 s ($P < 0.05$). From the 5th session onward, there were no significant changes in the time spent to complete the task. Until the 5th session there had been 1 or 2 heifers per session that did not complete the task within 2 min, but from the 6th session onward all the heifers accomplished the task (Figure 2). The heifers' age and the interactions between RTH and age had no significant effect.

As reported in Table 3, the average time spent by the heifers to touch the target 3 times was significantly affected by the RTH and the number of the session: NC heifers spent on average 7 s (32.6%) more than C and N heifers to accomplish the task ($P = 0.004$). The time to accomplish the task significantly decreased from the first to the 5th session ($P < 0.001$) and then stabilized (Figure 2 and Table 3).

As reported in Figure 3, on the first day of training 22%, 36% and 50% of C, N and NC heifers, respectively, retreated when offered the positive reinforcement (feed) upon accomplishing the task, and needed a negative reinforcement (temporary removal of the operator) as a reward. From the second day onwards, C heifers did not need negative reinforcement anymore, whereas N and NC heifers had to wait until the 6th and 7th session, respectively.

The willingness of heifers to be touched on the muzzle is shown in Figure 4, expressed as the proportion of heifers that allowed the operator to touch them on the muzzle, without retreating. Although C heifers were generally always more tolerant toward being touched on the muzzle than N and NC heifers, the difference between the RTH groups was significant only until the second session. The percentage of C heifers that allowed to be touched ranged from 100 to 60% compared with 45 - 86% of N and 11 - 40% of NC heifers, respectively.

The last 4 conditioning sessions aimed at letting the heifers get used to be touched on the rump and perineum and gently grabbed at the tail. All the heifers, while being target trained by the trainer, allowed R&P by a second operator, without leaving. Table 4 shows that the time over which heifers allowed R&P increased from the first to the 4th session, with a significant difference ($P < 0.05$) between the first and the second. Young heifers allowed to be touched for a longer time ($P < 0.05$) compared with old heifers, whereas no significant differences were found between RTH classes.

Table 3: Effect of age, responsiveness to humans (RTH) and their interactions on time spent to complete target training

		Time, s
Age	Old	24.2 (16–79)
	Young	22.2 (16–46)
Temperament	Confident	20.1[b] (16–33)
	Neutral	21.8[b] (17–48)
	Non-confident	28.5[a] (21–79)
Old	Confident	21.8 (18–33)
	Neutral	23.1 (16–48)
	Non-confident	28.2 (21–79)
Young	Confident	18.7 (16–31)
	Neutral	20.3 (17–34)
	Non-confident	28.8 (31–46)
SEM[1]		1.08
Probability	Age	0.279
	RTH	0.004
	Age x RTH	0.631
	Number of sessions	<0.001

[a, b] Means with different superscripts differ ($P < 0.05$).
[1]Variability is represented as standard error of means (SEM). Furthermore, ranges are reported in brackets.

As reported in Table 5, C heifers had lower ADT than N and NC ($P < 0.001$). ADT was not affected by training, but it was by period, in fact, in the second period, ADT significantly decreased compared with the first ($P < 0.001$). The interactions between factors were not significant with the exception of RTH × Period ($P < 0.001$), in fact the reduction of ADT between the first and the second periods was significant only for N and NC heifers. Heart rate showed a significant difference only as regards age, where young heifers had higher HR than old ($P < 0.001$). The RMSSD showed significant differences as regards period and training. Overall, RMSSD resulted higher during the second period ($P = 0.001$) and in trained heifers ($P = 0.077$), as reported in Table 5.

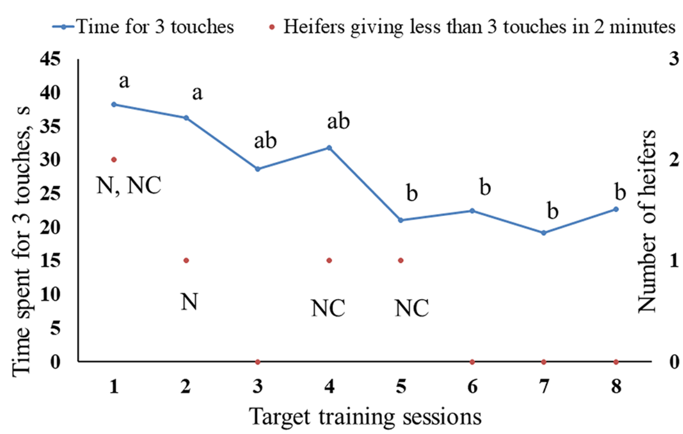

Figure 2. Time spent to complete target training in various training sessions in confident (C), neutral (N) and non-confident (NC) heifers. a, b Different letters indicate a significant difference ($P < 0.05$).

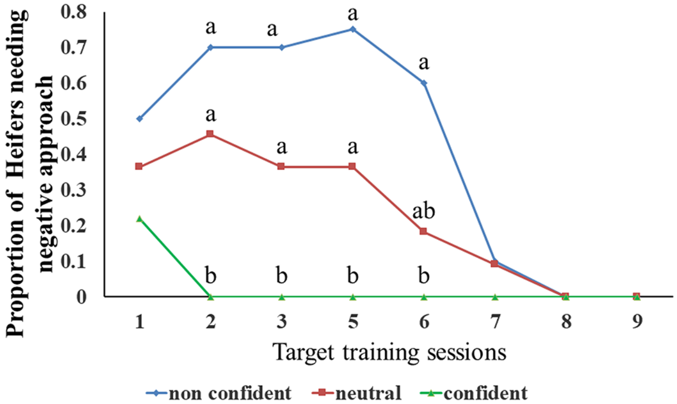

Figure 3. Negative approaches needed to complete target training session. a, b Different letters indicate a significant difference ($P < 0.05$).

Stress behavioral expressions, expressed as the risk of showing the tail clamped between hind legs, lowered head, lowered ears, eyes wide opened, stepping and kicking were never significant for any factor considered. On the whole, the average and (SD) of the above mentioned behaviors were 0.15 (0.36), 0.65 (0.48), 0.62 (0.49), 0.37 (0.48), 0.23 (0.43) at the beginning of the experiment 1 and 0.37 (0.48), 0.13 (0.34), 0.18 (0.39), 0.52 (0.50) and 0.17 (0.37) at the end, where the presence and the lack of the behaviors corresponded to 1 and 0, respectively. For brevity detailed data were not reported.

Figure 4 shows that FCM concentrations before and after handling were only affected by the heifers' RTH and not by age or training. C heifers had lower basal FCM levels than NC ($P = 0.04$), whereas N heifers showed an intermediate concentration. FCM concentrations after handling showed an opposite trend and were highest in C heifers followed by N and NC, respectively ($P = 0.003$).

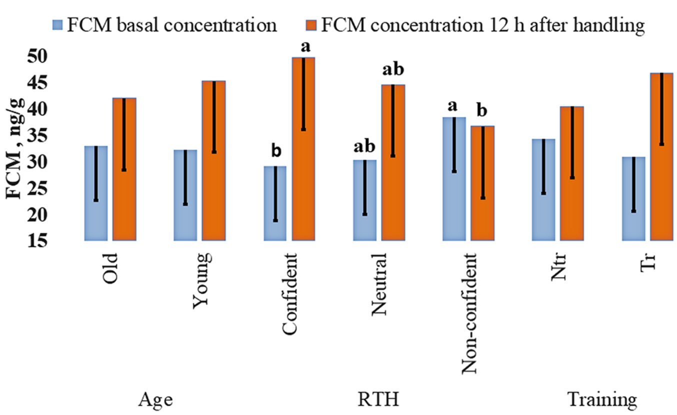

Figure 4. Effect of age, responsiveness to humans (RTH) and training on fecal cortisol metabolites (FMC): mean basal levels and concentrations measured 12 h after handling. Error bars indicate the standard error of means (SEM) a, b Different letters within variable, indicate a significant difference ($P < 0.05$).

Table 4. Effect of training session age and responsiveness to humans (RTH) on the time the heifers let to be touched on the rump and perineum

		Time, s
Session	1	9.20^{b} (0–15)
	2	13.2^{a} (0–15)
	3	14.2^{a} (0–15)
	4	15.0^{a} (15–15)
Age	Old	11.8^{b} (5–15)
	Young	14.0^{a} (7–15)
RTH	Confident	13.5 (11–15)
	Neutral	13.2 (7–15)
	Non-confident	12.0 (5–15)
SEM[1]		2.27
Probability	Session	<0.001
	Age	0.010
	RTH	0.280

[a, b] Means with different superscripts differ ($P < 0.05$).

[1]Variability is represented as standard error of means (SEM). Furthermore, ranges are reported in brackets.

As reported in Table S2 (https://doi.org/10.5281/zenodo.10474723, Marchesini et al., 2024) heifers body weight was significantly affected by age and period, with higher weights reported for older heifers ($P < 0.001$) and the second period ($P < 0.001$).

DISCUSSION

In the present experiment, operant conditioning was applied in a commercial dairy herd to heifers of different age and RTH, to verify whether this technique could be useful in facilitating routine handling procedures and reducing stress. In addition, we assessed whether the application of this technique in a commercial dairy farm is feasible, in terms of time spent by the farmer.

Target training

Wrede et al. (2004) trained only 10 heifers to respond to an acoustic stimulus, and Dirksen et al. (2020b) conditioned only 5 heifer calves to use a latrine. In the present study, starting with target training for short sessions (max 2 min), has given satisfactory results, since all the 31 trained heifers were successful in accomplishing their task after 5 sessions, regardless of their RTH class, with an average time of 25.3 s per session. It is noteworthy that confident heifers never failed the task and that, among neutral animals, no one failed after the second training session, confirming that the starting reactiveness to humans strongly affects the outcomes of training. This is in line with what was reported by Kutzer et al. (2015) and is confirmed

Table 5. Effect of age, responsiveness to humans (RTH), training, period and their interactions on the avoidance distance test (ADT), heart rate and Root Mean Square of Successive inter-beat interval Differences (RMSSD)

		ADT, m	Heart rate, min^{-1}	RMSSD, ms
Training	Ntr	0.518 (0–2.0)	86.6 (65–115)	14.2[y] (2.76–28.8)
	Tr	0.480 (0–2.0)	83.7 (63–114)	16.9[x] (4.50–35.5)
Age	Old	0.520 (0–2.0)	80.9[b] (65–97)	16.8 (4.31–33.0)
	Young	0.477 (0–1.8)	89.4[a] (63–115)	14.3 (2.76–35.5)
RTH	Confident	0.180[c] (0–0.85)	85.0 (73–115)	15.9 (2.76–29.2)
	Neutral	0.464[b] (0–1.1)	84.3 (63–110)	14.7 (4.31–33.0)
	Non-confident	0.852[a] (0–2.0)	86.1 (67–102)	16.0 (4.43–35.5)
Period [1]	P1	0.825[a] (0–2.0)	85.8 (63–115)	12.9[b] (2.76–35.5)
	P2	0.173[b] (0–1.1)	84.4 (65–114)	18.2[a] (4.50–33.0)
P1	Confident	0.22 (0–0.45)	87.1 (74–115)	12.5 (2.76–25.0)
	Neutral	0.75 (0–1.05)	83.5 (63–102)	12.8 (4.31–25.0)
	Non-confident	1.52 (1.1–2.00)	85.9 (74–102)	14.1 (4.44–35.5)
P2	Confident	0.14 (0–0.85)	83.7 (73–114)	18.8 (4.50–29.2)
	Neutral	0.18 (0–1.10)	84.0 (65–110)	16.6 (11.7–33.0)
	Non-confident	0.22(0–0.66)	84.9 (67–99)	18.4 (5.16–32.3)
SEM[2]		0.046	2.13	1.62
Probability	Age	0.335	<0.001	0.271
	RTH	<0.001	0.770	0.735
	Age x RTH	0.282	0.986	0.629
	Period	<.001	0.157	0.001
	Training	0.400	0.173	0.077
	Training x RTH	0.562	0.632	0.579
	Age x Training	0.851	0.685	0.356
	Age x Training x RTH	0.209	0.454	0.267
	Training x Period	0.601	0.383	0.315
	RTH x Period	<0.001	0.343	0.491

[a, b] Means with different superscripts differ ($P < 0.05$).[x, y] Means with different superscripts differ ($P < 0.1$).

[1]P1 = Period 1, before the final handling; P2 = Period 2, during final handling.

[2]Variability is represented as standard error of means (SEM). Furthermore, ranges are reported in brackets.

by the 38% highest average time spent to accomplish the task by NC heifers compared with the others. NC heifers took a bit more time to decide if trusting the operator and touching the target, and to accept the feed reward after every touch. Overall, after the 5th training session, the average time to complete the target training did not change, since the animal needed a minimum time to see where the target was, touch it and receive reinforcement for 3 times. This means that the target training session could be reduced to 5 sessions, instead of 8, thus saving the farmer further time. This is in line with the outcomes from other authors who, albeit for different purposes and with different methods, found that training heifers to milking routine, through udder massaging, for over 30 sessions, did not lead to any further improvements in milk let down time and milk flow rate (Das and Das, 2004). Touching the target 3 times was not important per se, but it meant that the animal had reduced its reactiveness toward the operator enough to accomplish the task. Ferguson and Rosales-Ruiz (2001) succeeded in teaching horses to move into a trailer and Dai et al. (2019) reduced the loading time and mitigated the loading-related stress in meat horses, using target training associated with positive reinforcement. In horses, target training has also been used to teach them to voluntarily move their head, shoulders, or quarters to facilitate husbandry and veterinary practices (Carrol et al., 2022). It is important to notice that after the first 3 sessions (1.5 weeks), the headlocks were open, so the heifers had the chance to leave, but, instead, they chose to stay and complete the training. This is another evidence of the reduced reactiveness toward humans achieved by the trained animals from the beginning of the experiment.

Negative reinforcement during target training

At the beginning of the experiment, negative reinforcement had to be used in more than one third of N and around 70% of NT heifers, to successfully accomplish the task required by TT. This means that those heifers were so hesitant or afraid of humans that, upon touching the target, refused to have the feed reward from the hand of the operator. The chance that the refusal of the reward was due to a different attractiveness of the reward itself was ruled out, since the heifers were allowed to choose between different rewards, (pellets for calves, dry cow ration) and because, from the seventh session on, all the heifers accepted the positive reinforcement. The correct use of negative reinforcement with the most distrustful heifers was essential to successfully involve them in the training and avoiding to rule them out. As reported by von Kuhlberg et al. (2021), in fact, relying only on positive reinforcement with inexperienced and stressed animals, sometimes leads to the refusal of the feed reward, resulting in a lack of reinforcement. The use of negative reinforcement has been previously reported to lead to an increase in feed intake and favor the acceptance of positive feed reward in sheep (Fernandez, 2020). Furthermore, the combination of positive and negative reinforcement during conditioning was found to be more effective in rhesus macaques (*Macaca mulatta*), when compared with positive reinforcement alone, to train the animal to move into a selected cage, in response to a stimulus (Wergård et al., 2015).

Training to muzzle touch

The training sessions aimed at further reducing the reactiveness of heifers toward the trainers, through conditioning heifers to be touched on the muzzle, failed. In fact, after 6 sessions there was no improvement in the percentage of heifers touched by the trainer, for any of the RTH classes. Allowing to be touched on the muzzle likely requires a very high level of willness to approach a human which might be reached after very long conditioning periods. However, since achieving that level of willness of contact in farm animals is not a priority, we concluded that this type of conditioning is not worth while doing.

Training to be touched on the rump and perineum

After 4 sessions.all the heifers, while being target trained, accepted to be touched on the rump and perineum (R&P) for 15 s, that was the maximum pre-set time per session, by a second operator who simulated the approach used in routine operations. The fact that heifers decided to stay, despite being touched on the rump and perineum instead of leaving, confirmed that conditioning reduced their reactiveness toward operators. It also represents an important achievement from the practical point of view, since for most of the sampling and routine operations, it is necessary to approach the heifers from the rear while they are at the feed bunk. Having animals that remain calm when approached helps avoiding possible kicks and injuries to the operators and makes the procedures feasible, even in farms lacking of headlock for heifers. Touching the rump, perineum and grabbing the tail is not as annoying and painful as taking blood, collecting feces from the rectal ampulla or performing artificial insemination, but training heifers to this kind of interactions makes them more used to the direct contact with operators. A similar result was also found by Lomb et al. (2021), by training heifers to subcutaneous injections through sham injections. The lack of differences between RTH classes in the time the heifers underwent R&P was likely due to the fact that these sessions were performed as last, and therefore, the conditioning effect of the previous sessions had led all heifers to have lower reactiveness toward the operators. The longer times over which the young heifers underwent the touch, compared with those older, are in line with some observations according to which the level of responding to test and training decrease with age. Nonetheless, these findings are not yet consistently demonstrated (Waiblinger et al., 2006).

Training time requirement

Given the results obtained and assuming that a farmer has 100 heifers and wants to condition them all, it would be convenient distributing the time dedicated to conditioning over 6 d a week. In this way, it would be possible maintaining a frequency of 2 sessions per heifers a week, for the first 3 weeks (target training), and then reducing the frequency to once a week for the following 4 R&P sessions. The daily time required by the farmer to condition the heifers for the first time would be on average of approximately 14 min a day for the 3 weeks of target training (25.3 s × 100 heifers × 2 sessions / 6 d / 60 s per minute), and then 4 min a day (15 s × 100 heifers × 1 session / 6 /60s per minute) for the next 4 weeks for R&P. Although such a procedure can be considered time-consuming, a 14-min daily time might be acceptable, if it eases the handling of the heifers for clinical visits, blood and fecal sampling, artificial insemination, growth measurements, etc.

Other studies on cattle conditioning required different durations to successfully accomplish the aimed task, according to the complexity and the aversiveness of the task itself, and the conditioning method used. The number of sessions required ranged, for example, from 10 to 30 to train dairy heifers to milking (Das and Das, 2004; Kutzer et al., 2015, von Kuhlberg et al., 2021). It took an average of 10.4 training sessions to respond to an acoustic cue and go to the feeder (Wredle et al., 2004), whereas conditioning calves to urinate in latrines, through different steps, required on average 44 trials (Dirksen et al. 2020b). Furthermore, conditioning heifers to undergo sham injection required up to 85 sessions using positive reinforcement training (Lomb et al., 2021). Compared with other studies, we obtained the achievement of the required tasks in a relatively short time. Among the possible reasons for this result, we have to consider that the required task was not very complex, nor painful. Training was performed without moving heifers in a different pen, thus reducing the stress that can be possibly aroused by changing the environment and isolate the trained heifers from all the others. The choice of using target training may have made the heifers gain confidence, as they could exercise control over events and choose whether or not having the reward. Furthermore, the initial use of negative reinforcement, for the heifers refusing the positive feed reward, allowed a relatively quick recruitment of the more reluctant heifers, who, with positive reinforcement alone, would have likely made progress very slowly, as reported by Lomb et al. (2021).

Effects of training on the Avoidance Distance Test (ADT)

According to the experimental design, by definition, confident heifers had lower ADT values than those neutral and non-confident. The reduction of ADT at the end of the experiment, especially for N and NC heifers was due to the increased confidence acquired during the training sessions for trained heifers and, likely, for the habituation to the presence of the staff for non-trained heifers. Unexpectedly, in fact, even NTr heifers reduced their ADT all the same. The latter result is likely due to non-associative learning, such as habituation, and social learning (Mellen & Ellis, 1996). Lomb et al. (2021), for example, reported that habituated heifers required a lower time to be pushed into a headlock than naïve heifers. Furthermore, cattle, as gregarious animals, tend to imitate or adapt to the behavior of the other individuals of the group, probably also due to a learning capacity by imitation. In support of a role for social learning in cattle, a study by Munksgaard et al. (2001) observed that cows observing another specimen receiving a positive handling tended to reduce the distance from the operator. This outcome suggests that the response of the observerving cows may be influenced by the response of those treated. In this regard, Colusso et al. (2020), in a study on virtual fencing, reported that when a cow received the acoustic stimulus and responded correctly by turning around, it triggered the same response in the surrounding cows.

Confident heifers did not show significant improvements in the ADT reduction, since they had already very low values at the outset. On the contrary, as regards the neutral and unconfident heifers, the ADT values decreased, demonstrating that heifers habituated to stay in close contact with humans.

Effects of training on Root Mean Square of Successive inter-beat interval Differences (RMSSD)

The increase of RMSSD, at the end of the experiment, indicates a lower level of stress compared with the beginning, and confirms that, overall, the heifers, over the experiment, have been desensitized toward proximity to humans and handling procedures. Higher values of RMSSD, in fact, indicate vagal activation and, therefore, greater relaxation (Wierig et al., 2018). This means that operant conditioning can reduce the activation of the sympathetic nervous system and consequently the impairment of the sympathovagal balance (Doerfler et al., 2016). Such an achievemet goes beyond the simple ADT reduction obtained through habituation to the presence of the personnel. An increase in RMMSD values are in fact reported following adaptation to a stressor (Doerfler et al., 2016) which, in our case, was represented by the presence of the personnel and the handling. Heart rate alone, as already reported by Kovács et al. (2015), it is not very valuable in assessing stress.

Effects of training and responsiveness to humans on Fecal Cholesterol Metabolites (FCM)

To investigate possible stress in heifers, the concentration of glucocorticoid metabolites in feces was analyzed as well. The higher baseline stress level in NC heifers indicates that these heifers were less able to cope with the housing environment, compared with confident heifers. This is typical of animals with a high responsive temperament (Sutherland et al., 2012). Heifers are in fact subjected to multiple stressors, such as human-animal interaction and hierarchical competition within the group. In our case, for example, competition for feed could have also been exacerbated by the presence of several headlocks, at the feed bunk, lower than the number of heifers. The association of ADT to the baseline FCM, found in our study, contrasts with the outcomes reported by Ebinghaus et al. (2020), who did not find any relationship. However, Ebinghaus and colleagues analyzed cows coming from 26 different farms, characterized by different facilities and management routines. The effect of farm and those other factors involved on FCMs could have easily masked the effect of ADT. Although ADT, is generally reported to accurately assess responsiveness to, or fear of humans (Kutzer et al., 2015; Waiblinger et al., 2006), it is logical to think that, since in a farm, animals always undergo a more or less direct interaction with humans, NC heifers are more stressed than others. Since basal FCM concentrations were measured at the beginning of the experiment, as expected, conditioning did not affect

FCMs, because when the samples were collected, the training sessions had not yet started.

The higher FCM levels found in C and N heifers, after handling, compared with NC heifers, was due to an increase of FCMs compared with basal values, which was not found in NC heifers. The handling performed at the end of the experiment represented our stressor after training. Although it cannot be considered a very strong stressor, it was strong enough to rise FCM in those heifers that had lower FCM basal values. This could be likely due to a decreased responsiveness of the adrenal gland to a stressor, in heifers in which the hypothalamic-pituitary-adrenal axis is activated more frequently. This was reported by Curley et al. (2008) in the beef cattle highly responsive to a challenge, which were also characterized by higher baseline cortisol concentration. Sutherland et al. (2012) reported that low responder cows, which had lower basal blood cortisol values compared with high responder cows, when milked in a novel environment, showed an increase of cortisol. On the contrary, high responders showed a decrease. The same authors did not find any differences in cortisol level between low and high responder cows after an ACTH challenge.

Behavioral changes

Although the handling at the end of the experiment was a stressor strong enough to elicit some changes as regards FCMs and RMSSD in some heifers, it did not elicit a consistent behavioral response. The behavioral expression of stress is not consistently reported in the literature, and can depend by the type and the strength of the applied stressor. Kutzer and colleagues (2015) found that, during first milking events, trained heifers stepped and kicked less often, had a lower likelihood of showing lowered ears, clamped tail and eyes wide opened, compared with those untrained. An opposite trend was reported by Eicher et al. (2007), whereas Sutherland et al. (2012) reported that cows experiencing increased stress for being milked in a novel environment did not display higher flinching, stepping or kicking activities. The same authors suggested that those behaviors could be associated with former negative handling experience.

Body weight changes

In our experiment, the slightly higher stress experienced by NC and Ntr heifers did not lead to significant differences in the final body weight. Possible reasons are the short period taken into consideration, the fact that the weight was indirectly estimated, and thus less accurate, and that the stressor applied to the animals was mild. Training per se has not been previously reported to increase the performance of cows trained before first milking (Kutzer et al., 2015; von Kuhlberg et al., 2021), whereas the influence of RTH in heifers' performance is not consistent in the literature. Kutzer and colleagues (2015) did not find any differences in milk yield related to the initial level of fear of humans, whereas Hemsworth et al. (2000) found a negative correlation between ADT and milk yield across several farms. On the contrary, Sutherland et al. (2012) reported that cows with a larger human avoidance distance showed a less disrupted milk let-down. With regard to body weight, Bacher et al. (2021) found that limousine bulls with lower avoidance distance at the feed bunk had heavier predicted 120-d and 400-d weights. The above differences in performance are likely due to the high number of factors affecting milk yield and body weight that makes the influence of ADT on performance context dependent.

Overall, this study demonstrates the successful desensitization of groups of heifers to the human presence and handling through operant conditioning involving target training. Training of part of the heifers reduced the vagal tone of trained animals and the avoidance distance to humans in both trained and not-trained heifers. The latter became more confident through non-associative learning, such as habituation. Training through operant conditioning resulted feasible since overall it required few minutes a day to the farmer. Non-confident heifers required more time to learn their tasks compared with the others and, in the first training sessions, they mostly required positive reinforcement being replaced by negative reinforcement. Notwithstanding the positive results, this experiment leaved some open questions. The learned tasks in cattle may be at risk of fading away if not periodically reinforced. However, in our case, whether or not routine movement and handling by the farmer are sufficient to maintain the learned behavior is a matter that requires further investigation. There are also unsolved questions about the minimum proportion of heifers that need to be trained to facilitate the habituation of all the others to the human presence and handling. Additionally, it remains to be explored whether or not operant conditioning aimed at desensitizing heifers to handling can even reduce stress during group changes.

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