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Effect of *pre-partum* habituation to milking routine on behaviour and lactation performance of buffalo heifers

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ABSTRACT

The aim of this study was to investigate the effect *pre-partum* habituation in the milking parlour on behaviour and lactation performance of buffalo heifers. Sixteen buffalo heifers in late gestation were used for this study. The animals, with an age ranging from 30 to 44 months at the start of the study, were equally allocated into two treatments and balanced for estimated calving date. Eight animals received *pre-partum* habituation for 10 days before the estimated calving date (group H), while the eight others received no treatment and served as the control (group C). During the *pre-partum* habituation sessions, group H animals were moved to the milking parlour and left for 10 min in the milking stall once each day, where the udder was thoroughly washed with warm water, wiped with a disposable towel and massaged. The behaviours, registered from entrance into the milking stall to exit, were step and kick. After calving milk flow profiles, milk yield and milk quality variables were determined along with behavioural recordings. The H animals during the habituation procedure showed a reduction in the number of steps ($P < 0.001$) and kicks ($P < 0.01$). After calving the H animals performed fewer steps than the control animals at 0 ($P < 0.001$), 3 ($P < 0.01$), 6 ($P < 0.01$), 13 ($P < 0.01$) and 20 ($P < 0.01$) days after calving. Animals from group H also performed fewer kicks than control animals at 0 ($P < 0.001$), 3 ($P < 0.01$), 6 ($P < 0.01$) and 13 ($P < 0.01$) days after calving. In the C group a reduction in the number of steps ($P < 0.001$) and kicks ($P < 0.001$) was observed as lactation proceeded, whereas for group H only a tendency for a reduction over lactation was detected. *Pre-partum* habituation did not significantly affect milk quality or milk flow variables. Milk yield in the first 3 min of milking ($P < 0.001$), and average milk flow ($P < 0.001$), increased throughout the experimental period, whereas the duration of the pre-milking phase decreased as lactation proceeded ($P < 0.001$). This study shows that buffalo heifers exposed to a *pre-partum* habituation programme performed fewer steps and kicks than control animals during milking. Therefore, it is concluded that using this treatment can reduce the level of restlessness in buffalo heifers during milking.

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1. Introduction

In Italy, buffaloes (*Bubalus bubalis*) are used as dairy animals. Their milk is mainly used to make mozzarella cheese

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and, in recent years, a substantial increase in dairy buffalo farming has taken place (Cavallina et al., 2008; De Rosa et al., 2009; Caria et al., 2011). A similar increase in buffalo farming is taking place in many developing countries such as India, Pakistan and China where the buffalo has become an important milk-producing animal (Thomas et al., 2004; Borghese and Mazzi, 2005).

It is hypothesised that buffalo have a high sensitivity to milking because they have only recently been introduced to both machine milking and early calf separation practices that are commonplace with other dairy animals (Napolitano et al., 2013).

Contact with a novel environment, and contact with humans, can induce stress responses (Hemsworth et al., 1989; Arnold et al., 2007) and reduce productivity (Rushen et al., 1999a; Sutherland and Huddart, 2012) in dairy cows. The milking routine can affect animal welfare, and a change in this routine may cause stress during milking (Munksgaard et al., 2001; Rushen et al., 2001). Buffaloes are very consistent in their milking routine (Polikarpus et al., 2014) and are sensitive to changes in the environment; when they are uncomfortable they may withhold their milk (Borghese et al., 2007). In dairy cows up to 20% of the milk in the udder is stored in the cistern (Bruckmaier and Wellnitz, 2008). Buffaloes have a small udder cistern and only 5% of the milk is stored there, the rest of the milk is stored in the alveoli and small ducts (Thomas et al., 2004). The alveolar milk ejection depends on released oxytocin concentration and myoepithelial contraction (Bruckmaier and Blum, 1998; Bruckmaier, 2005; Bruckmaier and Wellnitz, 2008). If animals are stressed adrenaline is secreted, and this may reduce the supply of oxytocin through vasoconstriction or by blocking the oxytocin receptors localised on the myoepithelial cells of the udder alveoli (Thomas et al., 2008). Therefore, buffaloes in a stressed state are more likely to have impaired milk letdown than dairy cows under similar stress conditions. When buffalo milk ejection is disturbed, stockmen commonly use exogenous oxytocin in order to allow the complete evacuation of milk from the udder (Saltamacchia et al., 2007; Neglia et al., 2008; Cavallina et al., 2008), but this procedure can result in reduced milk ejection (Bruckmaier, 2003).

Heifers' milk ejection may be further hindered by neophobia, as they are exposed to several novel stimuli associated with the milking routine. This can result in disturbed milk ejection (Van Reenen et al., 2002), increased restlessness during milking (Cavallina et al., 2008), increased risk of injury for the milkers (Willis, 1983), increased incidence of mastitis for the animals (Ivemeyer et al., 2011) and reduced welfare (Sutherland and Huddart, 2012). Different *pre-partum* strategies have been proposed to mitigate these detrimental effects, such as positive handling (Hemsworth et al., 1989), exposure to tape-recorded milking facility noise (Arnold et al., 2007) or familiarization with the milking parlour (Bremner, 1997; Das and Das, 2004). Although several investigations on the *pre-partum* habituation of heifers in the milking parlour have been conducted on dairy cattle (Bremner, 1997; Das and Das, 2004; Sutherland and Huddart, 2012), no studies are available on dairy buffaloes.

Therefore, this study aimed to investigate the effect of *pre-partum* milking habituation on behaviour, milk production and milk-flow profile of buffalo heifers.

2. Materials and methods

2.1. Farm and animals

The research was conducted from February to May on the experimental farm of the Campania Regional Council. The farm was located in southern Italy on the Sele Plain (40°36′ N, 15°3′ E). The animals were selected from an artificially inseminated herd of 40 heifers according to their estimated calving dates (conventional gestation length = 310 d). The 16 heifers with the closest estimated calving dates (over a 2-week period) were used. The animals, with an age ranging from 30 to 44 months at the start of the study, were equally allocated into two treatments, balanced for the estimated calving date. Eight animals received *pre-partum* habituation for 10 days before the estimated calving date (group H), while the eight others received no habituation and served as the control (group C). The two groups were kept in separate indoor pens, with a space allowance of 10 m^2 per head. They also had free access to an outdoor loafing area with an earth floor. Each day, at 08:00 h, all subjects were offered a complete mixed diet ad libitum. The milking parlour, a 2 × 3 auto-tandem, was equipped with the ALPRO® system (De Laval, Sweden). This system allows the collection of data from the milking parlour, including the milking position of each cow, milk yield, time and duration of milking. The herd was milked twice a day (05:30 and 17:00) by the same two stockpersons throughout the experiment.

2.2. Behavioural recordings

2.2.1. Pre-partum habituation phase

During the *pre-partum* habituation sessions, group H animals were moved to the milking parlour and remained for 10 min in the milking stall once a day (at the end of the afternoon milking). During this time, the udder was thoroughly washed with warm water, wiped with a disposable towel and massaged by a stockperson. The massage included stripping of the teats. Behavioural observations were performed at the first day of entrance into the milking parlour and then at 3-day intervals until calving. For subsequent statistical analysis only the first three habituation sessions (day 0, 3 and 6) were used, because calving occurred within 8–15 days (median = 12.5 d) after the start of habituation and only five animals calved after the day 9 of habituation phase.

The behaviours, registered from entrance into the milking stall to exit, were step (whenever foot was lifted from the ground but less than 15 cm) and kick (foot raised above 15 cm off of the ground and kick given in any direction) performed with hind legs as they are considered signs of agitation in cattle and buffaloes (Munksgaard et al., 2001; De Rosa et al., 2005). Although, height was used as discriminant, animals rising their legs above 15 cm usually express this behaviour more energetically, thus facilitating the observer task. Defecation, urination and vocalisation were recorded and combined as a single activity, as suggested by Rushen et al. (2001). A trained observer located in the pit continuously recorded the behaviour of the animals. The variables were expressed as number of occurrences per minute.

2.2.2. Post-partum milking phase

Heifers from both groups after calving were left in the home pen with their calves and brought into the milking parlour within 12 h of calving. They were then moved, without the calf, into the same mixed group comprising H and C cows as well as primiparous and multiparous non-experimental cows. With all the animals combined (experimental + non-experimental), there were 36 buffaloes, with a space allowance of 18 m^2 per head. Behaviours in the parlour were observed during the first milking, and then on seven afternoon milkings (on days 3, 6, 13, 20, 27, 41 and 55 after calving). Udder preparation and behavioural recordings from entrance into the milking stall to the removal of the milking cluster, were the same as those performed during the *pre-partum* habituation. Heifers showing no sign of milk release after 90 s from the attachment of the milking cluster were injected intramuscularly with 2 ml (20 International Units) of exogenous oxytocin (Ipofamina, Laboratories Biové, Arques, France). Any behavioural reactions at the time of oxytocin administration (i.e. during the injection) were not recorded as they could have been a direct response to the puncture, then the observation continued as scheduled because possible subsequent effects of the injection were considered negligible in comparison with the general response of the animals to the milking procedures. One heifer from group H suffered uterine prolapse soon after calving. This animal was not included in the subsequent recordings or statistical analyses.

2.3. Milk flow profile, milk yield and milk quality

Milk flow profiles and milk quality variables were determined on seven occasions (days 3, 6, 13, 20, 27, 41 and 55 after calving) along with behavioural recordings. Milk flow profile variables were measured with an electronic mobile milk-flow-metre Lactocorder (WMB, 2005). The following variables were recorded: total milk yield (kg); milk yield in the first 3 min (kg); duration (min) of whole milking (from attachment to cluster removal); duration (min) of pre-milking phase (from cluster attachment to a milk flow $\geq$0.5 kg/min), duration (min) of increasing phase (from milk flow rate >0.5 kg/min until the start of the plateau phase), duration (min) of plateau phase (phase of steady flow); duration (min) of decreasing phase (from the end of the plateau phase to milk flow >0.2 kg/min); duration (min) of main milking phase (sum of increasing, plateau, and decreasing phases, i.e. starting with a milk flow $\geq$0.5 kg/min and ending with a milk flow $\leq$0.2 kg/min); duration (min) of overmilking phase (from the end of the main milking phase to cluster removal with a milk flow $\leq$0.2 kg/min); maximum milk flow (kg/min); mean milk flow (kg/min, calculated during the main milking phase). Subsequently, individual milk samples were withdrawn from the sample bottles attached to individual milking units and placed in 40-ml plastic tubes. Samples were analysed for lactose, fat and protein contents (IDF, 1990) using an infrared spectrophotometer (Milko Scan 605; Foss Electric, Hillerød, Denmark) and for somatic cell count (IDF, 1995) using a Somacount 300 (Bentley Instruments, Chaska, USA).

2.4. Statistical analysis

Parametric analyses were performed using SAS software (1990), whereas non-parametric analyses were conducted with SPSS software (2009). The animal was used as the experimental unit. The combined activity of defecation/urination/vocalisation was observed so rarely that it was not included in the statistical analysis. For each variable the assumptions of parametric tests were checked before data analyses. The Shapiro–Wilk's test across the levels of each factor was used to test the normality of distribution, and for each factor homogeneity of variance was assessed with Levene's statistic. The numbers of steps and kicks recorded during the behavioural observations violated the assumptions of parametric analyses and transformation of the original data also infringed these assumptions. Therefore, nonparametric tests were used for the analysis of these variables. The numbers of steps and kicks recorded during *pre-partum* habituation were analysed with the Friedman test, using days of habituation (0, 3 and 6) as factor. When significant effects were found, the differences between days were evaluated by the Wilcoxon test. For each day after calving, the numbers of steps and kicks were analysed with the Mann–Whitney test using treatment (T and C) as factor. For each group the Friedman test was used with days after calving (0, 3, 6, 13, 20, 27, 41 and 55) as factor. All other variables recorded after parturition were analysed with a linear mixed model, with treatment (T and C) as non-repeated factor, and days after calving (3, 6, 13, 20, 27, 41 and 55) and treatment × time as repeated factors. Heifer variance was considered to be random and was used as the error term to test the main effect of treatment. Eleven milk flow profiles derived from oxytocin-injected animals, and the corresponding data for milk quality, were excluded from statistical analysis. Logarithmic transformation was used to normalise the skewed nature of the somatic cell count data. Where appropriate, the Tukey test was used to identify differences between least squares means. Comparison of the prevalence of oxytocin injection at milking between the treatments (number of oxytocin injections used during the behavioural observations/number of observed milkings) was carried out with the χ^2 test.

3. Results

Heifers from group H showed a reduction in the number of steps (χ^2 = 15.06, df = 2, P < 0.001) and kicks (χ^2 = 10.75, df = 2, P < 0.01) per minute over the habituation period (Table 1).

The number of steps and kicks per minute expressed by habituated and control animals throughout the experimental milking period are shown in Table 2. Group H heifers performed fewer steps per minute than the control animals until day 20 after calving. Animals from group H also performed fewer kicks than control animals until day 13

Table 1
Median (min–max) number of steps and kicks (n/min) expressed in the milking parlour by buffalo heifers during habituation before calving.

	Day of habituation phase		
	0	3	6
Steps, n/min	0.25 (0.1–0.8)[A]	0.15 (0.1–0.3)[BC]	0 (0–0.1)[BD]
Kicks, n/min	0.50 (0–0.7)[A]	0.20 (0–0.4)[aB]	0.10 (0–0.2)[Bb]

Within a row (a, b) indicate a significant difference at $P<0.05$. Within a row (A, B and C, D) indicate significant differences at $P<0.01$.

Table 2
Effect of *pre-partum* habituation on median (min–max) number of steps and kicks (n/min) expressed in the milking parlour by habituated (H) and control (C) animals after calving.

Days after calving	Step, n/min		U[a]	Significance	Kick, n/min		U[a]	Significance
	H	C			H	C		
0	0.10 (0–0.29)	0.59 (0.30–1.77)	0	***	0.20 (0–0.27)	0.83 (0.40–1.82)	0	***
3	0.09 (0–0.18)	0.25 (0–1.25)	7.5	**	0.08 (0–0.20)	0.56 (0–1.08)	6	**
6	0(0–0.10)	0.26 (0–0.75)	7	**	0(0–0.09)	0.34 (0–0.54)	4	**
13	0(0–0.09)	0.20 (0–0.45)	4	**	0(0–0.09)	0.27 (0–0.45)	8	**
20	0 (0–0)	0.13 (0–0.27)	7	**	0 (0–0)	0.06 (0–0.36)	14	NS
27	0 (0–0)	0(0–0.22)	17.5	NS	0 (0–0)	0(0–0.27)	17.5	NS
41	0 (0–0)	0(0–0.19)	21	NS	0 (0–0)	0(0–0.10)	24.5	NS
55	0 (0–0)	0(0–0.10)	24.5	NS	0(0–0.08)	0 (0–0)	24	NS

NS = not significant.
[a] Statistic used in the Mann–Whitney test.
** Differences between treatments are significant at $P<0.01$.
*** Differences between treatments are significant at $P<0.001$.

after calving. In group C a reduction in the number of steps ($\chi^2=37.50$, df = 7, $P<0.001$) and kicks ($\chi^2=39.56$, df = 7, $P<0.001$) was observed as lactation proceeded, whereas for group H only a tendency was found ($\chi^2=12.91$, df = 7, $P=0.0745$ and $\chi^2=12.69$, df = 7, $P<0.001$; for steps and kicks, respectively). During the 24 *pre-partum* habituation sessions no animals vocalised, whereas during the 120 *post-partum* milking sessions one control animal vocalised once on the first milking and another control animal vocalised twice on the same occasion.

Pre-partum habituation did not significantly affect milk flow variables and milk quality. In addition, no significant interactions between treatment × days after calving were found. For the sake of brevity, only the principal milk flow variables are reported in Table 3. As expected, milk yield ($F_{6,67}=11.16$, $P<0.001$), milk yield in the first 3 min of milking ($F_{6,67}=4.64$, $P<0.001$), and mean milk flow ($F_{6,67}=4.15$, $P<0.001$) increased throughout the experimental period as a consequence of the progression of lactation. The duration of the pre-milking phase decreased as lactation proceeded ($F_{6,67}=6.78$, $P<0.001$). Days after calving also induced a significant decrease in milk protein ($F_{6,67}=10.25$, $P<0.001$) and milk fat concentrations ($F_{6,67}=7.61$, $P<0.001$) and somatic cell count ($F_{6,67}=3.65$; $P<0.001$), whereas lactose content ($F_{6,67}=16.79$, $P<0.001$) increased with time after parturition.

Table 3
Effect of *pre-partum* habituation on milk flow and milk quality variables (least square mean ± standard error) of habituated (H) and control (C) animals.

	Treatment		Significance[a]
	H	C	
Total milk yield, kg/milking	3.64 ± 0.21	4.19 ± 0.29	NS
Milk yield in the first 3 min, kg/milking	2.85 ± 0.35	3.19 ± 0.31	NS
Duration of whole milking[b], min	7.18 ± 0.56	8.46 ± 0.46	NS
Duration of pre-milking phase[c], min	1.83 ± 0.28	1.95 ± 0.22	NS
Duration of main milking phase[d], min	3.61 ± 0.32	3.83 ± 0.26	NS
Duration of overmilking phase[e], min	2.09 ± 0.35	2.63 ± 0.25	NS
Max milk flow, kg/min	1.65 ± 0.17	1.58 ± 0.15	NS
Average milk flow[f], kg/min	0.94 ± 0.09	1.02 ± 0.08	NS
Protein, %	4.45 ± 0.15	4.52 ± 0.13	NS
Fat, %	8.05 ± 0.26	8.14 ± 0.22	NS
Lactose, %	4.54 ± 0.07	4.42 ± 0.06	NS
Somatic cell count, n/ml	130,059 ± 19,684	115,009 ± 16,759	NS

[a] NS = not significant.
[b] Period of time from attachment to cluster removal.
[c] Period of time from cluster attachment to a milk flow ≥0.5 kg/min.
[d] Period of time starting with a milk flow ≥0.5 kg/min and ending with a milk flow ≤0.2 kg/min.
[e] Period of time from the end of main milking phase to cluster removal with a milk flow ≤0.2 kg/min.
[f] Calculated during the main milking phase.

The prevalence of oxytocin injections did not differ between treatments. During the two months of lactation there were four oxytocin injections out of a total of 56 milkings (7.1%) and seven out of a total of 64 milkings (10.9%) for groups T and C, respectively (χ^2 = 0.17, df = 1, NS). For group H the four injections were given to two animals during their first milking and the third day of lactation. For group C the seven injections were given to four animals during their first milking and then on three of the previous animals on the third day of lactation.

4. Discussion

Animal restlessness at milking is a possible source of injury (Hemsworth and Coleman, 2011). Stepping and kicking during milking may be caused by many different factors such as pushing of adjacent cows, lameness, low mineral intakes, presence of haematophagic insects, or poor maintenance of the milking machinery (Saltamacchia et al., 2007). However, these behavioural expression are usually interpreted as indicators of agitation both in cattle (Hemsworth et al., 2000; Munksgaard et al., 2001) and in buffaloes (De Rosa et al., 2005). Overall, the results indicate that *pre-partum* habituation can be effective as a means to reduce stepping and kicking, and thus behavioural restlessness among buffalo heifers in the milking parlour. Machine milking also involves psychological factors (e.g. negative behaviour of the stockperson, calf separation, unfamiliarity to the milking parlour), which may increase heifer agitation. However, as the habituated heifers were observed to step and kick significantly less than the control cows during the first 20 days *post-partum*, this indicates that the habituated heifers were less affected by the milking procedures in early lactation than those that had had no habituation. Therefore, neophobia was likely to be the primary aspect involved in the induction of buffalo heifer agitation. The further finding that the control animals showed a reduction in these behaviours with increased time exposure to the milking parlour routine supports the hypothesis that unfamiliarity to these procedures may adversely affect their response. Similar results have been reported for cattle (Bertenshaw et al., 2008; Sutherland and Huddart, 2012; Ivemeyer et al., 2011). In buffaloes, Cavallina et al. (2008) compared the behaviour of primiparous and multiparous cows in the milking parlour in early lactation and found that primiparous animals kicked more frequently compared to multiparous animals and also that only primiparous cows showed a significant reduction of kicking over time, which suggests that it could be associated with the animal's milking experience. Habituation is a non-associative form of learning defined as the gradual decrease in agitation symptoms of a response due to repeated stimulation to novel harmless situations in the absence of reinforcement and punishment (Price, 2008). In the case of buffalo heifer habituation in the milking procedures, increased stockperson safety and animal welfare may be expected as a consequence of reduced animal restlessness. In fact, a reduced incidence of stepping and kicking has been associated with reduced milk cortisol and improved handling of dairy cattle (Hemsworth et al., 1989).

The few vocalisation occurrences observed in our study were also reported for dairy cows: Rushen et al. (1999b) rarely recorded vocalisations when cows were milked in presence of aversive or gentle handlers, whereas Rushen et al. (2001) recorded a reduced number of vocalisations in animals milked in their home stall or in an isolation chamber.

In cattle, Sandrucci et al. (2007) found that good udder preparation can result in better milking performances, such as higher milk yield per milking and a shorter total milking time, compared with poor udder preparation. Similarly, in buffaloes Bava et al. (2007) suggest that appropriate pre-milking stimulation and prompt cluster removal are necessary in order to ensure optimal milk letdown. In our study both experimental groups received the same udder preparation before milking.

In the current study milk flow variables and milk quality were affected neither by treatment nor by a treatment × time after calving interaction. These results may be interpreted as a discrepancy with behavioural response recorded during milking. However, responses to environmental challenges are complex and varied and behavioural indicators are not necessarily correlated with physiological responses (Dawkins, 2003). Das and Das (2004) report that *pre-partum* udder massaging not only reduced the level of fear in primiparous cows but also reduced milking time by facilitating a quick letdown of milk and a higher rate of milk flow. These different results may be attributed to the different animal species considered and to the length of the treatment, which in the study of Das and Das (2004) consisted in a higher number of habituation sessions distributed over a longer period of time and a higher duration of each session. As expected, milk yield, milk yield in the first three minutes of milking, and mean milk flow increased throughout the post-calving period, whereas the duration of pre-milking phase decreased. Others have found a decrease in mean milk flow rate over the whole of lactation, and this would be expected, as the milk flow rate curve follows the characteristics of the lactation curve (Bava et al., 2007; Sandrucci et al., 2007). However, in the current study only the first 55 days of lactation were considered. And so the increase in mean milk flow rate observed over this early period is in accord with expectations.

A significant effect of time after calving on milk protein, fat and lactose contents, as well as on the somatic cell count, confirm the expected trend for these variables in other dairy species over the course of lactation.

In a previous study (Saltamacchia et al., 2007) the prevalence of exogenous oxytocin injections in buffaloes ranged from 5.9 to 46.5% per milking (median = 9.5%), thus similar to that observed in this study. However, the use of oxytocin injections during the trial period, albeit not significantly, was lower in those heifers that had had *pre-partum* habituation. Our results suggest that habituation can decrease indicators of stress and restlessness like kicks and steps, but does not improve milk flow and quality. In addition, frequent treatments with exogenous oxytocin to speed up milk ejection in buffaloes seems unnecessary and related to improper milking practices and poor human–animal relationship (Saltamacchia et al., 2007).

5. Conclusions

This study shows that buffalo heifers exposed to a *pre-partum* habituation programme performed fewer steps and kicks than control animals during milking. Conversely, *pre-partum* habituation affected neither milk flow profiles, milk yield nor the milk quality of buffalo heifers. It can be concluded that *pre-partum* habituation in the milking parlour can reduce the level of restlessness in buffalo heifers during milking in early lactation.

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