Impact of a dietary citrus extract on the behavior and milk yield of lactating dairy cows following regrouping

by
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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Animal Bioscience

Guelph, Ontario, Canada

ABSTRACT

EFFECT OF CITRUS EXTRACT ON REGROUPED COWS

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University of Guelph, 2021

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The objective of this study was to determine if feeding a citrus extract (CE; from *Citrus sinensis*) reduces the effect of regrouping on lactating dairy cows. Thirty-two cows were enrolled and housed in a tie-stall facility where they were fed a diet with 4 g/d of CE or not. Cows stayed for 7 d in the tie-stall facility, then moved to 1 of 2 free-stall pens (each containing 29 other cows) for 7 d. Primiparous cows fed CE had sustained rumination and lying time after regrouping and had greater feeding time, greater milk yield, lesser idle standing time, and initiated fewer aggressive interactions after regrouping compared to primiparous control cows. For multiparous cows there was little effect of CE on their response to regrouping. The results indicate that feeding a CE additive to mid-lactation primiparous dairy cows may have mitigated the negative effects of regrouping on behavior and milk yield.

ACKNOWLEDGEMENTS

I would like to first thank my advisor Dr. Trevor DeVries, for taking me first as visiting student back in 2016, giving me an opportunity to work with research in Canada and second as a graduate student, I will always be grateful to you for giving me those opportunities. I will be forever grateful for all your advice, fast answers and review on my thesis. After working a few years with you, now I can say that I worked with one of the best researchers in the world.

Secondly, I would like to thank my advisory committee member, Dr. Renée Bergeron, for all her input and suggestions. I really appreciate your time, smart inputs and fast reviews of my thesis.

I would also like to thank the staff of the Elora Dairy Research and Innovation Centre for making this project possible, taking my feed samples and being always willing to help me when I needed. A special thanks to Jeff McFarlane for always agreeing to help me, even when we had to spend 4 hours passing a cable underneath mats full of manure and to Laura Wright for all her advice and assistance moving my cows and planning the regrouping schedule.

Thank you to all the members of cowcrew. Since the moment that I was picked up at the airport on my first visit to Canada, you all have been amazing and made me always feel welcome, supported and liked. It has been really fun (except for 2020). First, I would like to thank Meagan, you were really helpful with my trial and always fun to work with and have been one of my craziest best friends since I got here; I truly miss our days on the farms. Thank you Brooke for all the help with data and videos, I'll miss hanging out with you. Anna and Robert thank you for all the help setting up the cameras at the beginning of the trial. Thank you, Sarah and Bruna, for climbing up the ladder for me when I was away, that was really brave of you. Thank you for all the volunteers that also helped me collect data, that made my job a lot easier. My old but gold cowcrew friend Sydney, even though you couldn't help me much in my trial (conveniently always busy on the day that I needed help), you were always there for me, invited me into your house and made me feel like your family was my family. I am truly so lucky to have you as a friend. Casey, although I enjoy annoying and giving you a hard time, I want you to know that I really appreciate how good you have been to me, you are a really fun and kind person, love you.

I would like to give a special thank you to the best partner a person could ask for, Kaitlyn Dancy. You have been helping me since the first day that I arrived in Canada. You went above and beyond helping me, watching basically all the social and feeding behavior videos, grinding my feed samples, helped me with stats and endless grammar questions, always being so patient and kind with me; I don't think I could've done this without you. I can sure say that the 4 years that you made me wait to be with you was worthy. Now endless new adventures await us in Nova Scotia, and I'm excited to go to the next chapter of my life with you. Last but not least, um enorme agradecimento para a minha mae que sempre acreditou em mim e me apoiou. Sem a sua bencao, conselhos, e suporte eu nao estaria aqui, voce e a pessoa mais fantatisca e persistente que ja conheci na vida, uma inspiracao para todos ao seu redor. Ao meu pai, minha irma e toda minha familia (Helena, Saira, tios, tias e primos), seu amor e carinho me fizeram a pessoa que sou hoje, amo voces.

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LIST OF ABBREVIATIONS

ADF – acid detergent fiber

BCS – body condition score

BW – body weight

CE – citrus extract

DM – dry matter

DIM – days in milk

DMI – dry matter intake

HPA – hypothalamic-pituitary-adrenal

NDF – neutral detergent fiber

NEFA – greater plasma nonesterified fatty acids

TMR – total mixed ration

TDN – total digestible nutrients

SAM - sympathetic-adrenal-medullary

CHAPTER 1: GENERAL INTRODUCTION

Dairy cows are typically grouped according to level of milk production, parity, DIM, and body condition score (Contreras-Govea et al., 2015). Grouping of dairy cows has been suggested to be an effective method to decrease animal heterogeneity, therefore, increasing cow productivity, animal health, animal welfare, and farm profitability (Grant and Albright, 2001). As result of on-farm management, dairy cows may be regrouped up to 4 to 5 times during a single lactation (von Keyserlingk et al., 2008). Regrouping may cause psycho-social stress on cows (Chen et al., 2015), disturbing their productive behaviors (i.e. rumination, lying and feeding time) and milk yield (Phillips et al., 2001; von Keyserlingk et al., 2008; Schirmann et al., 2011). Researchers have demonstrated that competition between cows is increased when they are regrouped to new pens, re-establishing their social hierarchy through physical and nonphysical interactions (Bøe and Færevik, 2003). Feed bunk competition can decrease dry matter intake (DeVries et al., 2003), milk production (von Keyserlingk et al., 2008), and may have other negative impacts on lying and rumination behaviour (von Keyserlingk et al., 2008; Schirmann et al., 2011).

Given the negative impacts of regrouping, alternatives to reduce its undesirable impacts need to be identified. Management strategies, including housing cows in adjacent groups (i.e. so cows that are about to be regrouped may have some familiarity to the future new pen mates, potentially reducing the negative effects of regrouping), not regrouping cows to overstocked pens, and housing multiparous and primiparous separately (Grant and Albright, 2001; von Keyserlingk, 2005; Mazer et al., 2020) may be used to decrease the negative effects of regrouping. Alternatives to reduce the negative impacts of regrouping may include these various management strategies, as well as the use of sensory feed additives, which have demonstrated effects on reducing stress responses in animals. For example, Val-Laillet et al. (2016) demonstrated that pigs that were fed a transition diet with a food ingredient manly composed with citrus extract (CE) had a moderate preference for the post transition diet that had the CE. Those authors proposed that the pigs supplemented with the food ingredient had showed different brain responses in areas responsible for reward anticipation and perception (i.e. sites that may be impaired during chronic stress), suggesting that CE may be beneficial to animals undergoing stressful events, such as regrouping. However, to our knowledge, the effects of CE on cow production and behavior experiencing regrouping have never been investigated.

This literature review will firstly discuss the criteria used to group cows (i.e. nutrient requirements, milk production, parity, DMI, etc.) and how regrouping affects cow production, behavior, and welfare. Finally, this review will discuss mitigation of the effects of regrouping, including the use of sensory additives.

1.1 Grouping strategies and the effects of regrouping on cow stress, behavior, milk production and hierarchy

1.1.1 Grouping strategies and cow productive behaviors

The grouping of dairy cows may have a large influence on dry matter intake (DMI), milk production, and animal welfare. Effective cow grouping decreases competition at the feed bunk and may increase DMI (Grant and Albright, 2000). A homogeneous group can also improve feed efficiency and decrease nutrient excretion (Cabrera et al., 2012) by facilitating targeted nutrient intake relative to nutrient requirements (Barrientos-Blanco et al., 2020). To take advantage of those findings, researchers have recommended that dairy farms should have no more than 4 lactating cow groups (McGilliard et al., 1983; St. Pierre and Thraen, 1999). Grant and Albright

(2001) outlined 9 main factors to consider when determining the optimal group size in dairy farms, including: feed bunk space and competition for feed, availability of water and beds, and social interactions among cows and how they are affected by group size, space availability to the cow, size of holding area and capacity of milking parlor (for conventional milking parlours), animal body size and age, body condition, DIM, stall size and uniformity (i.e. stalls equally comfortable within group to decrease fighting over more comfortable stalls), and proper ventilation. Grouping cows in competitive environments (i.e. overstocked stalls and/or pens, insufficient space per cow at the feed bunk) may increase aggressive behavior, restricting access to desired resources (i.e. free stalls and feed bunk) (von Keyserlingk et al., 2008), and thus decrease animal welfare (von Keyserlingk et al., 2009).

Behaviors such as rumination, lying time, and feeding time are essential for milk production maintenance (DeVries and von Keyserlingk, 2005; Kaufman et al., 2018; Johnston and DeVries, 2018). Cows housed in free-stall barns typically divide their day into 3 to 5 h/d eating, consuming 9 to 14 meals, ruminating 7 to 10 h/d, spending 30 min/d drinking water, 2 to 3 h/d being milked (Grant and Albright, 2000), and at least 12 h/d of lying and resting time (NFACC, 2009). Regrouping has been reported to negatively affect lying time (von Keyserlingk et al., 2008), rumination (Schirmann et al., 2011), and feeding time (Phillips et al., 2001). Regrouping also increases agonistic behaviors between cows (von Keyserlingk et al., 2008; Schirmann et al., 2011) and decreases milk production (von Keyserlingk et al., 2008). Those negative effects of regrouping on the behavior and milk production of cows may be due to an increased psycho-social stress (Chen et al., 2015).

1.1.2 Stress

Stress can be generally defined as any event that disturbs the constant homeostatic state of the animal (Goldstein and Kopin, 2007). Cows may experience stress during several occasions in their lifespan in response to environmental and husbandry management events such as regrouping, dry off, heat stress, maternal separation and culling (Flower and Weary, 2003; von Keyserlingk et al., 2008; Bertulat et al., 2013). Animals may respond to disturbances of the homeostatic state by showing aggression against a threat or fleeing away (Baldwin, 2013; Chen et al., 2015). Psychological or physical factors have been described as contributors that can disturb the homeostatic state of animals (Chen et al., 2015). Further, those authors defined psycho-social stress as a psychological stress response triggered by social events (i.e. regrouping, weaning, restraint), disturbing the homeostatic state of the animal. Mammals undergoing stressful situations may have an internal integrated response to stress, signaling the two distinct axes (i.e. hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM)) (Minton, 1994). The hypothalamus is responsible for initiating the stress response in the HPA axis by liberating hormones (corticotrophin releasing hormone and vasopressin), those hormones then send a signal to the pituitary gland to liberate adrenocorticotropic hormone (Minton, 1994). Further, the release of glucocorticoids from the adrenal cortex is initiated as endocrine response of stress. Psychological contributors may cause cows stress, therefore activating the SAM axis, triggering a fast response of the animal and initiating aggression or fleeing behavior (Chen et al., 2015).

1.1.3 Aggressive behavior

Regrouping events have been reported to increase aggressive interactions between cows regrouped and their pen mates (von Keyserlingk et al., 2008). Competitive behaviors, such as displacement at the feed bunk, are more likely to happen at feeding time (Olofsson, 1999; DeVries and von Keyserlingk, 2006). Displacements can be described as when a cow (actor) pushes or head-butts another cow (reactor) from the feed bunk or stalls resulting in a complete withdraw by the reactor (DeVries et al., 2004). Further, displacements are more likely to happen in confinement housing systems than in grazing systems (O’Connell et al., 1989; Black and Krawczel, 2016). This may be due to cows having more space in grazing systems, therefore, making it easier to avoid aggressive interactions by fleeing to another location. In a regrouping study conducted with dry cows it was reported that cows doubled the number of displacements from the feed bunk on the first day after regrouping compared with their baseline period when introduced into a new group (Schirmann et al., 2011). von Keyserlingk et al. (2008) reported that cows increased the number of displacements in the feeding area on d 1 and 2 after regrouping compared with their baseline. This is consistent with other authors who have reported that regrouping increases competition between cows at the feed bunk (Grant and Albright, 2001). Regrouped cows had their competitive displacements from the lying area reduced on the first day after regrouping in the von Keyserlingk et al. (2008) study. These researchers speculated that focal cows were unwilling to dispute their favorite stalls with their new pen mates. On the other hand, Fregonesi et al. (2007) described that cows displaced their pen mates seeking access to their preferred stalls, even though they had free access to other stalls. Thus, further research is required to better understand what motivates cows to engage or not in agonistic behaviors in regrouping scenarios.

1.1.4 Feeding, rumination time, and DMI

Researchers have reported that cows that have greater feeding time are also more likely to have greater milk production (Johnston and DeVries, 2018). The stress associated with regrouping may decrease feed consumption (Grant and Albright, 2001). Phillips et al. (2001) reported that grazing time of multiparous and primiparous cows kept on pasture was reduced when they were regrouped in the same outdoor pen compared with multiparous and primiparous cows that were regrouped in two other outdoor pens sorted by parity. Those researchers suggested that cows had overall greater aggressive interactions after regrouping due to the intensified competition for better grass quality, decreasing the grazing time of the cows.

Time spent ruminating reflects rumen health, as well as the overall level of feed consumption and milk yield (Clément et al., 2014; Kaufman et al., 2018). Changes in rumination behavior can lead to and indicate several health issues in dairy cattle such as acidosis, metritis, ketosis, displaced abomasum (Stangaferro et al., 2016a; Kaufman et al., 2018). Schirmann et al. (2011) observed that dry cows that were regrouped to a new pen had decreased rumination time and DMI on d 1 compared with their baseline and that their time spent ruminating returned to the baseline on d 3 following regrouping. In contrast, Hasegawa et al. (1997) reported that regrouping did not present negative effects on rumination time of cows. However, they started to record rumination time 2 days after regrouping and, as reported in Schirmann et al. (2011), the effect of regrouping may start to decline on d 2.

Researchers have reported that DMI may not change after regrouping, suggesting that even under feed competition cows may adapt their feeding behavior by increasing meals/d with shorter periods to maintain DMI (Grant and Albright, 2001; Val-Laillet et al., 2008). Although there are a few studies demonstrating that DMI might remain stable after regrouping, Schirmann et al. (2011) detected that non-lactating cow had decreased DMI on d 1 after regrouping due to stressors of the new environment. This suggests that more research is needed to better understand the effects of regrouping on DMI on lactating dairy cows.

1.1.5 Lying behavior

Several researchers have demonstrated that lying behavior affects cow health, productivity, and welfare (Solano et al., 2016; Westin et al., 2016). Further, lying behavior has the highest priority for cows as compared to their other behaviors (Munksgaard et al., 2005). Regrouping has been reported to decrease lying time as result of an increase in aggressive interactions between cows introduced to the new group and their pen mates (von Keyserlingk et al., 2008). Talebi et al. (2014) reported that cows spent less time lying when they were regrouped in an overstocked pen; that may have been due to increased competition at the stall, preventing cows from accessing their preferred stalls.

1.1.6 Social grooming

Allogrooming is a form of social licking that animals perform; it is part of maternal care and frequent among growing and adult cattle (Schloeth, 1961; Kiley-Worthington and de la Plain, 1983). Researchers have described that social grooming may decrease tension in animals under stressful situations (Aureli and de Waal, 1997). von Keyserlingk et al. (2008) investigated how regrouping cows individually influenced allogrooming, by introducing focal cows individually to a new pen with unfamiliar cows. Although those researchers were expecting that allogrooming would increase after regrouping, due to allogrooming being assumed to decrease social tension and regrouping being a type of psycho-social stress, these authors rather reported that allogrooming decreased after regrouping. Given that animals may need prior bonding to perform allogrooming (Aureli and de Waal, 1997), the cows in the von Keyserlingk et al. (2008) study may not have had that with any cows in the new pen. de Freslon et al. (2020) demonstrated that older cows groomed more other individuals as compared with younger cows, and those grooming events were more directed to cows of a similar age to themselves, suggesting that seniority may be a factor associated with social grooming. Val-Laillet et al. (2009) suggested that primiparous cows have their allogrooming decreasing due to those cows being more susceptible to show a lack of socio-positive interactions (i.e. allogrooming and preferential spatial associations) when exposed to competitive environments and events, such as regrouping. That suggests that cows that have allogrooming and grooming negatively affect may have more difficulties coping in great competitive events. Regrouping multiparous and primiparous cows in the same pen has been reported to increase competition between parities (Neisen et al., 2009; Mazer et al., 2020), to our knowledge there is no research to date that has demonstrated that regrouping events affected allogrooming negatively in primiparous cows after regrouping. Further studies are needed to investigate if allogrooming in primiparous cows is negatively affected by regrouping.

1.1.7 Milk Yield and health

A few researchers have investigated how regrouping may negatively affect milk yield in regrouped cows. Although in early studies it was reported that milk production was not affected by regrouping (Clark et al., 1977; Sowerby and Polan, 1978), these studies had limited sampling methods (i.e. regrouped several cows at the time in commercial farms). Alternatively, several researchers have reported that cows had decreased milk production after regrouping (Brakel and Leis, 1976; Hasegawa et al., 1997; von Keyserlingk et al., 2008). Although the decrease in milk yield after regrouping in these studies only lasted a few days (i.e. suggesting that the effects of regrouping on production is temporary), those regrouped cows had a significant amount of milk yield decreased in the d 1 (3.7 kg) after regrouping (von Keyserlingk et al., 2008). Greater competition at the feed bunk and reduced DMI after regrouping is the probable cause for cows decreased milk yield. Torres-Cardona et al. (2014) reported data from 500 lactating cows, demonstrating that primiparous cows had decreased milk yield after regrouping as compared with their baseline (2 days before regrouping). However, these researchers did not present sufficient evidence (e.g. no measurement of change in feed intake) to explain their findings.

The acute negative effects of regrouping on behavior, production, and DMI may not necessarily affect cow health. Studies by Silva et al. (2013a,b) tested the effect of 2 prepartum grouping strategies (i.e. one treatment had a stable group with no cows added to the group, whereas the other treatment cows were regrouped one at the time weekly to the pen) on health and productive parameters. Interestingly, peripartum blood NEFA and glucose concentrations, and postpartum β-hydroxybutyrate and energy corrected milk were not different between treatments, suggesting that maintaining a stable group did not improve cow health and production (Silva et al., 2013a,b). These findings suggest that regrouping may have a short and acute effect on behavior and production, but these return to baseline levels a few days after regrouping (von Keyserlingk et al., 2008) and do not necessarily have longer-term impacts.

1.1.8 Social hierarchy

Magee and Galinsky (2008) defined social hierarchy as the arranged ranking of individual animals within groups, with different level of dominance, with animals experiencing competitive success or defeat over some things they desire. Cows use their social hierarchy mainly to allocate limited or desired resources (i.e. food, lying stalls, water) (Kondo and Hurnik, 1990). Boe and Faerevik (2003) reported that cows initiate aggressive interactions to establish social hierarchy and that dominance hierarchies can be rapidly established. Those researchers also observed that once dominance was established, the agonistic behavior between cows decreased, suggesting that hierarchy may be stable and does not alter over time. Further, researchers have demonstrated that cows that are regrouped may take 24 to 72 h to re-establish their social hierarchy (Moran and Doyle, 2015). Body weight, age, lactation number, chest girth, and body length to be the main traits that may affect dominance rank (Stricklin et al., 1980; Brantas, 1968; Beilharz and Zeeb, 1982). Hasegawa et al. (1997) measured the dominance hierarchy of primiparous heifers after regrouping. Interestingly, those researchers demonstrated that moving dominant heifers to a new pen decreased the dominance rank that they had prior to being moved. Moreover, the remaining dominant and middle rank heifers had their dominance rank increased, demonstrating that regrouping may affect social hierarchy, disturbing not only the introduced animal, but also the resident animals. Heifers who had experienced a reduction in their dominance rank following regrouping had decreased milk production (Hasegawa et al. 1997), likely due to the increased agonistic behavior they faced, which in turn may have reduced their DMI following regrouping. Furthermore, in a beef cattle study, introduced animals were more stressed by social integration problems than resident animals, due to increased agonistic behavior initiated by the resident cows (Mench et al., 1990).

Early research demonstrated that dominance relationships in cows may have a linearly hierarchy within group (e.g. A is dominant over B and B is dominant over C and A is also dominant over C) (Schein and Fohrman, 1955). However, Beilharz and Mylrea (1963) reported that cows may have triangle relationships in their hierarchy, suggesting that dominance ranking can be categorized in high (dominant), middle and low (submissive) ranking cows with no linear order. In regrouping events, dominant middle ranking animals may take more longer adapting to their new pen than dominant high and low ranking animals (Krohn and Konggaard, 1980).

Researchers have recommended separating primiparous from multiparous cows in different pens (Grant and Albright, 1995; Phillips and Rind 2001), as mixing them may decrease primiparous milk production and disturb their feeding behavior (Bach et al., 2006). Primiparous cows are typically in a lower position in the social dominance hierarchy as compared to multiparous cows (Wierenga, 1990), and have been demonstrated to exhibit reduced DMI and milk production when placed in the same pen as multiparous cows (Phelphs et al., 1992). In early research, it was reported that primiparous cows, when regrouped apart from multiparous cows, spent more time eating/d (10-15%), had greater DMI/d (17-18%), greater meal bouts/d, and greater milk production/d (4-16%) (Konggaard and Krohn, 1978). Primiparous cows also had greater fecal cortisol metabolites when introduced individually to a new group of cows as compared with multiparous cows that were also introduced individually to a new group of cows (Mazer et al., 2020).

1.2 Reducing the negative effects of regrouping

Given the negative effects of regrouping, it is important to seek alternatives, which may include management strategies, as well as the use of sensory feed additives.

1.2.1 Management strategies

Alternatives to reduce physical and nonphysical interaction among cows include housing cows in adjacent groups, allowing limited contact before moving them to a new pen and moving them in groups rather than individually, considering that management procedures are more stressful for an isolated cow (Grant and Albright, 2001). Boyle et al. (2012) compared the effects of regrouping cows in the morning versus at night on behavior and production. Those researchers detected that cows who were regrouped in the morning experienced more overall aggression in the overall month (4 observations total) after regrouping than cows regrouped at night. These results indicate that regrouping cows at night, rather than in the morning, may be beneficial. This may be due to the fact that cows may have reduced agonistic behavior in the evening, spending most of the night lying down and ruminating (Nakanishi et al., 1993; Johansson et al., 1999;

Boyle et al., 2012). Further research analyzing potential effects of regrouping heifers in the evening, rather in the morning, with a larger sample size is needed, as Boyle et al. (2012) only used 6 heifers in their study.

Regrouping primiparous and multiparous separately may reduce competition within pen (i.e. feed bunk, lying stall, alley, etc.), which may reduce the negative effects of regrouping on behavior and milk production. As compared to heifers regrouped with younger cows, heifers regrouped with older cows had greater lying time (Konggaard and Krohn (1978), as cited by Bøe and Færevik, 2003), probably due to heifers failing to compete for feed from the feed bunk with multiparous cows and lying down instead. O'Connell et al. (2008) demonstrated that introducing heifers in pairs rather than individually to a pen with multiparous cows, reduced lying behavior and increased standing time immediately after regrouping. These authors also reported that those heifers that were regrouped in pairs had greater milk yield and had more hoof lesions 3 months after they were regrouped than the heifers that were regrouped individually. O'Connell et al. (2008) suggested that their findings were due to heifers regrouped in pairs having greater behavioral activity during the first month after regrouping than the heifers that were regrouped individually. Further, Neisen et al. (2009) investigated the effects of regrouping single and pairs of heifers known to each other into a new pen, and demonstrated that heifers introduced to a new pen individually received more aggressive interaction than heifers introduced in pairs (7.19 aggressive interaction/h vs 3.79 aggressive interaction/h). Schirmann et al. (2011) analyzed the effects of regrouping cows in groups of 3 non-lactating cows to a new pen and detected that the frequency of aggressive behavior and displacements between cows did not change after regrouping as compared with the aggressive behavior displayed 7 days before regrouping. Schirmann et al., (2011) suggested that their findings on aggressive behavior were mitigated by their study design (i.e. regrouped cows in the afternoon and regrouped cows in groups). Further, Tesfa (2003) investigated the effect of regrouping cows (a pair of cows at the time) on milk yield and agonistic behavior. Although those researchers did not detect a reduction in milk yield of cows, they detected that agonistic behavior of cows had increased on the first day of regrouping and tended to decrease in the following 2 days after regrouping, suggesting a negative effect of regrouping on cows. Additionally, Mazer et al. (2020) did not detect any differences in fecal cortisol metabolites and behavior (i.e. lying, feeding and social) between multiparous and primiparous when they were regrouped along with a familiar partner, suggesting that regrouping cows in groups rather than individually did not show any benefit. The overall findings suggest that some management alternatives to reduce the negative effects of regrouping on cows (regrouping cows in pairs) have to be further investigated, due to conflicting results between studies.

Regrouping cows to a new pen and increasing its stocking density may disturb the social hierarchy within pens, and may lead to increased aggressive competition between cows, especially during feeding time (von Keyserlingk, 2005). Greater pen stocking density can increase competition at the feed bunk, at the lying stall, and overall, in the pen (DeVries et al., 2004; Huzzey et al., 2006; von Keyserlingk et al., 2008), thus, increasing aggressive behavior between cows in the pen. Researchers have demonstrated that a mixed group of primiparous and multiparous cows in an overstocked environment had greater plasma NEFA, fecal cortisol concentrations, and plasma glucose (Huzzey et al., 2006). These findings suggest that dry cows had worse health and more stress indicators when experiencing feeding and lying competition in overstocked pens than cows placed in pens that were not overstocked. Fregonesi et al. (2007) studied lying behavior in overstocked cows and demonstrated that cows that were overstocked at

150% (cows/stalls) spent 1.7 h/d less time lying down than cows that were not overstocked (at 100% overstocking). Further, those researchers reported that cows that were overstocked at 150% had a latency to lie down after milking that was 13 min shorter than the cows that were not overstocked. Some cows in overstocking situations may have experienced a type of indirect competition and preferred to lie down after milking to secure a place in the stalls rather than go eating (Fregonesi et al., 2007). However, Fregonesi et al. (2007) did not test for preference between free stalls vs feed bunk after milking, thus it is uncertain if those cows went to the lying area by choice or because they were prevented to access the feed bunk and then decided to lie down while waiting.

The use of headlocks on farms has been reported to decrease competition among cows at the feeding bunk (Bolinger et al., 1997). Endres et al. (2010) evaluated the effects of two types of feed-line barriers, headlocks and post-and-rail, on the feeding and social behavior of dairy cows. They suggested that cows that had access to the feed through headlocks had fewer numbers of aggressive displacements due to headlocks allowing subordinate cows to have better access to the feed than with a post-and-rail barrier. Thus, feeding cows through headlock barriers can be an alternative to decrease agonistic interactions at the feed bunk after regrouping.

1.2.2 Supplementation of sensory additives

There may be other opportunities to reduce the negative impacts of regrouping, including the use of sensory feed additives. Individuals supplemented with sensory feed additives such as phytogenic (i.e. spices and herbs) and flavor feed additives may have greater feed intake and growth due to greater feed palatability (Windisch et al., 2008; Franz et al., 2010; Jacela et al., 2010). Moreover, sensory additives such as citrus extract may also be used to mitigate stress in animals (Menneson et al., 2020).

Citrus extract supplementation has been demonstrated to have positive effects, including reducing anxiety situations (Lehrner et al., 2005; Faturi et al., 2010) and potentially increasing palatability and feed acceptance (Clouard and Val-Laillet, 2014) in individuals undergoing stressful. In a study with humans it was reported that ambient odor of orange decreased anxiety and increased calmness in patients waiting for dental care (Lehrner et al., 2005). Clouard and Val-Laillet (2014) demonstrated that CE led to increased palatability and acceptance of solid feed in piglets during the early postweaning period, suggesting a positive effect of these additives in piglets, reducing anxiety and increasing appetite under stressful conditions. Further, in a study with Wistar rats, it was reported that an exposure to sweet orange aroma (*Citrus sinensis*) reduced anxiety in the rats (Faturi et al., 2010). Those researchers tested 3 different levels of CE (100, 200, or 400 µl) and detected that all doses had anxiolytic activity at some level and that the highest dose had greater positive effects than the lower doses. On the other hand, not every type of citrus induces the same positive effects. Researchers have demonstrated that a bitter orange CE may have chemical compounds with appetite-suppressing properties for body weight control, whereas, a sweet orange CE feed additive may stimulate feed preferences on piglets (Astell et al., 2013).

Val-Laillet et al. (2016) reported that CE stimulated brain activation in the insular cortex, the amygdala, and the striatum (sites in the brain responsible for reward perception and anticipation) in piglets familiarized with it, suggesting that CE may have had a positive impact on piglets' appetite. CE was also tested to evaluate its potential anxiolytics effect in humans exposed to a stressful circumstance (Goes et al., 2012). Goes et al. (2012) demonstrated that individuals exposed to CE did not present significant alterations in their state-anxiety, tension, and tranquility level when subjected to an anxiogenic situation, indicating that CE may have anxiolytic properties. Menneson et al. (2020) reported that the olfactory stimulation in pigs fed CE led to mitigation of brain activity in sites related to acute stress (i.e. hippocampus, cingulate cortex, and areas associated with arousal), suggesting that CE supplementation reduced chronic stress in pigs undergoing psycho-social stress events. When tested in children undergoing a stressful situation (going to the dentist), *Citrus sinensis* essential oil reduced mean blood pressure, respiratory rate, and pulse rate (Pour et al., 2013), suggesting that the CE had a positive effect reducing anxiety in those children. Further, Faturi et al. (2010) reported reduced anxiety of rats subjected to CE under a stressful situation and the reduced anxiety effect exhibited in these rats may had been produced through the CE binding to olfactory receptors and initiate an electrophysiological response extending to the brain. Further, odor perception was activated by that response activating the neocortex (i.e. responsible for emotional responses). It was also supposed that activation of areas responsible for anxiolysis in the brain with CE inhalation led limonene (*Citrus sinensis* mainly compound) to the lungs then this compound was diffused into the blood and reached the brain via systemic circulation (Faturi et al., 2010). Researchers from different institutions have done two large reviews on studies that investigated the anxiolytics effects of CE (Dosoky and Setzer, 2018; Mannucci et al., 2018). Those researcher groups both concluded that individuals that inhaled or consumed CE and were exposed to stressful situations demonstrated some extent of reduced anxiety. These findings indicate that CE has positive effects reducing anxiety on individuals undergoing stressful situations.

Researchers have detected lesser SCC in dairy cows supplemented with CE and undergoing heat stress (Havlin and Robinson, 2015). Those authors suggested that the great amount of vitamin C in the CE was associated with better overall udder health. Further, Ying et al. (2017) tested if supplementation of CE to early lactation cows would increase DMI and milk production. Interestingly, these researchers did not detect any increase of DMI, and milk yield of those cows supplemented with CE. These findings suggest that supplementation with CE may produce greater benefits to dairy cows undergoing some type of stress, which did not happen in Ying et al. (2017) study. However, to our knowledge, CE has never been tested to mitigate the effects of a psycho-social stressor on dairy cows; thus, research is needed to investigate the potential effects of CE on cow behavior and production after a stressful event, such as regrouping.

1.3 Objectives and hypothesis

Regrouping effects are greater for those cows introduced into a group, rather than those already within the group (Mench et al., 1990). There is also agreement among researchers that aggressive behaviors will increase after regrouping due to the modification of social hierarchy in the group (Phillips et al., 2001; von Keyserlingk et al., 2008; Tesfa, 2013). As result, milk production and lying behavior will decrease for few days after regrouping, and then they will return to their baseline levels (von Keyserlingk et al., 2008). Feeding behavior can also decrease due to excessive competition at the feed bunk, preventing cows from approaching the feed bunk (Phillips et al., 2001). Thus, decreased feeding times may be associated with decreased DMI (Schirmann et al., 2012) as well as rumination time. Supplementation of CE has been shown to decrease anxiety in individuals undergoing stressful circumstances (Faturi et al., 2010). There is a potential for CE to lessen the psycho-social stress associated with regrouping and, thus, minimizing changes in behavior and sustaining milk production after regrouping. However, to our knowledge, there is no research to date where the effects of the CE on cows have been studied.

Thus, the objective of my thesis research was to determine if feeding a citrus extract (derived from *Citrus sinensis*) would reduce the negative impacts of social regrouping of lactating dairy cows on behavior and milk production. It was hypothesized that cows supplemented with CE would have less psycho-social stress as compared with control cows fed the control diet, due to the positive effects on productive and social behaviors. As a result, cows supplemented with CE were predicted to have greater milk yield than control focal cows after regrouping. It was also predicted that CE focal cows would demonstrate a lesser change in lying time and rumination time compared with their baseline than focal control cows after regrouping. It was also predicted that CE focal cows would have greater feeding time and lesser agonistic behavior than cows fed the control diet after regrouping. Finally, it was also hypothesised that primiparous cows would experience a greater regrouping effect than multiparous cows.

CHAPTER 2: IMPACT OF A DIETARY CITRUS EXTRACT ON THE BEHAVIOR AND MILK YIELD OF LACTATING DAIRY COWS FOLLOWING REGROUPING

2.1 INTRODUCTION

Dairy cows in commercial farms are frequently moved to form groups similar in age, stage of lactation, milk production, health, and reproductive status (Grant and Albright, 2001; Bøe and Færevik, 2003). It has been reported that cows may experience up to 4 to 5 regrouping events during any single lactation (Grant and Albright, 2001).

Researchers have previously suggested that mixing cows with unfamiliar animals, which already have an established social order, destabilizes the social dynamic within the group (Bøe and Færevik, 2003). After regrouping, dairy cows increase their level of physical social interactions and competition (Kondo and Hurnik, 1990; Bøe and Færevik, 2003). This increase in competition may result in reduced lying, feeding, and rumination behavior, leading to transitory decreased dry matter intake (DMI) and milk production (e.g. Phillips and Rind, 2001; von Keyserlingk et al., 2008; Schirmann et al., 2011). Torres-Cardona et al. (2014) also demonstrated that relocation may reduce milk production on the day after relocation, with a greater impact on first-lactation heifers compared with mature cows.

Given the negative impacts of regrouping, and its necessity within modern dairy farm management, ways to reduce these negative impacts need to be identified. This may be done through changes in farm management. For example, Talebi et al. (2014) demonstrated that the increases in competitive behavior in cows following regrouping and associated decreases in lying times may be mitigated by reducing pen stocking density. Further, Tesfa (2013) demonstrated that lactating cows introduced into new groups of cows as pairs, as opposed to individually, showed no drop in milk production as demonstrated in previous studies. More recently, Mazer et al. (2020) demonstrated that primiparous cows, when individually moved to a new pen after calving, exhibited greater fecal cortisol metabolite concentration in the days subsequent to regrouping as compared with primiparous cows moved along with a partner. Interestingly, those researchers also reported greater fecal cortisol metabolite concentration in primiparous cows than multiparous cows when introduced to a new group individually, suggesting that primiparous cows may be more sensitive to the negative effects of regrouping as compared with multiparous cows (Mazer et al., 2020).

There may be other opportunities to reduce the negative impacts of regrouping, including the use of sensory feed additives. Researchers have demonstrated that male Wistar rats experienced reduced anxiety after being exposed to a citrus extract (CE) essential oil derived from *Citrus sinensis* (Faturi et al., 2010). When tested in children, *Citrus sinensis* essential oil reduced mean blood pressure, respiratory rate, and pulse rate during a stressful situation (Pour et al., 2013). In work by Val-Laillet et al. (2016), it was demonstrated that feeding a *Citrus sinensis* based CE feed additive to pigs had positive impacts on brain activation in the insular cortex, the amygdala, and the striatum (putamen and caudate), suggesting that the CE stimulated reward perception and anticipation in pigs. Researchers have also demonstrated that CE decreased chronic stress in pigs undergoing psychosocial stress (Menneson et al., 2020). To our knowledge, no research to date has been conducted to evaluate the effects of a *Citrus sinensis* based CE on the response of dairy cattle to a potentially stressful situation.

The objective of this study was, therefore, to determine if feeding a CE (derived from *Citrus sinensis*) reduced the negative impact of social regrouping of lactating dairy cows on behavior and milk production. It was hypothesized that cows supplemented with CE in their diet would experience less negative effects on their behavior (feeding, ruminating, lying, and social) and milk production after being moved into a new group of cows, compared with cows fed a control diet. Additionally, it was hypothesized that CE supplementation would have a greater effect on primiparous cows as compared with multiparous cows.

2.2 MATERIALS AND METHODS

2.2.1 Animals and housing

Thirty-two lactating Holstein dairy cows, including 14 primiparous and 18 multiparous (parity = 2.0 ± 1.2; mean ± SD), were selected from the University of Guelph, Elora Research Station – Ontario Dairy Research Centre (Elora, Ontario, Canada) dairy herd for use in this study. Selected cows were in mid-lactation (169.8 ± 16.8 DIM) at the time of entry into the study and were producing, on average, 36.8 ± 6.2 kg/d of milk. Cows were kept in 1 of 2 free-stall pens (except for during the acclimatization period, see below), each containing 30 free-stalls and 2 water troughs. Stalls were mattress-based (Pasture Mat; ProMat, Woodstock, ON, Canada) and bedded with chopped straw. The 2 free-stall pens were 56.4 m apart. Each free-stall pen contained a stationary (non-mechanical) scratching brush. Cows were fed a total mixed ration (TMR) (Table 2.1), 1x/d, between 0930 and 1130 h. A feed refusal rate of 5% of offered feed was targeted. Cows were milked 2x/d (at 0430 and 1630 h). Animals were managed according to the standard operating procedures for this facility.

The use of cows and experimental procedures complied with the guidelines of the Canadian Council on Animal Care (2009) and were approved by the University of Guelph Animal Care Committee (AUP#4131).

2.2.2 Experimental design

Focal cows (n = 32) were individually assigned, balanced for parity (i.e. number of primiparous and multiparous in both treatments), to 1 of 2 treatments: 1) control TMR (control diet; n = 16; primiparous = 7; multiparous = 9), or 2) control TMR with 4 g/d of citrus extract (Phodé, Terssac, France) (CE diet; n=16; primiparous = 7; multiparous = 9). Control cows were fed the control TMR with a placebo (200 g/cow/d of wheat middlings) and CE cows were fed the control TMR with the feed additive (196 g/cow per day of wheat middlings mixed with 4 g/cow/d of citrus extract). To ensure that would not happen any cross-contamination of the treatments the experimental cows were fed in two different feed wagons (i.e. same make and model) and stayed in pens far away from each other. Focal cows (2 at a time) were moved from a non-experimental free-stall pen (different free-stall pen area than the experimental pens cows were later moved into) to a tie-stall area, where they were housed (and fed and milked) in tie-stalls where those focal cows were at least 15 m apart from each other. Other non-experimental cows were occasionally housed in tie-stalls between the focal cows. Tie-stalls were mattress-based (Pasture Mat; ProMat, Woodstock, ON, Canada) and bedded with chopped straw. Upon entry to that housing, cows were assigned to 1 of the 2 experimental treatment diets and fed those diets for 7 d in that tie-stall area. Dry matter intake (DMI) was recorded daily for these cows, based on feed offered and feed refused. After 7 d of feeding the treatment diets, focal cows were moved to 1 of 2 experimental free-stall pens right before feeding time (at 1030 h). At that time point, 2 more focal cows were moved into the tie-stall area. Cows in the experimental free-stall pens were fed the same diets as the cows that were moved into those respective pens (i.e. one pen was fed the control TMR with placebo [200 g/cow/d of wheat middlings], while the other pen was fed the control TMR with the feed additive mixture [196 g/cow per day of wheat middlings mixed with 4 g/cow/d of citrus extract]). Pen DMI were recorded 3 d per week based on feed offered and feed refused. Prior to the first 2 focal cows being moved into the free-stall pens, cows in those pens were fed their respective treatment diets for a 2-wk period to allow those cows establish social order and acclimate to those diets. Those pens each consisted of 30 lactating dairy cows in mid to late lactation (average across the study DIM = 265.02 ± 83.05 d; BW = 753.84 ± 76.75 kg; BCS = 3.19 ± 0.32; mean ± SD). Upon entry of the experimental focal cows to those pens, 1 late lactation cow from each pen was removed to make space for the incoming cow. The focal cows were monitored for a period of 7 d following regrouping in their new pen. After 7 d, the next 2 focal cows were moved into the free-stall pens, with again 1 late lactation cow from each group being removed to make space for the incoming cow. This process was repeated until all 16 cows per treatment had been introduced to the experimental free-stall pens.

2.2.3 Behavioral data collection

Standing and lying behavior. Standing and lying behavior data of the focal cows were recorded with electronic data loggers (HOBO Pendant G Data Logger, Onset Computer Corporation, Bourne, MA), as validated by Ledgerwood et al. (2010). Measurements were taken at 1-min intervals; leg orientation data was used to compute standing and lying duration. Data loggers were attached to the medial side of the hind leg of each cow using veterinary bandaging tape (Vetrap Bandaging Tape, 3M, London, ON, Canada) at entry to the tie-stall barn area. After 7 d (at time of regrouping), loggers were removed (and data extracted), and a new logger was placed on the other hind leg for an additional 7 d, after which the data was extracted. Data from these loggers were used for the analysis comparing the focal cow lying time (min/d) after regrouping with their own baseline measured before regrouping.

Rumination behavior. An electronic monitoring system (HR-TAG-LD, SCR Engineers Ltd., Netanya, Israel), as validated by Schirmann et al. (2009), was used in this study to monitor rumination activity of all focal cows, and all other non-cows in each experimental free-stall pen. Rumination data loggers were attached to a nylon collar that was fitted to each cow on the first day of enrolment to monitor rumination activity throughout the study period. Data were continuously uploaded to a control unit through a radio frequency reader. Raw data were stored in 2-h intervals and then combined into a continuous record to determine the total time spent ruminating each day for each cow.

Feeding, social, and grooming behavior. Feeding time, social behavior and grooming behavior of focal cows were determined from continuous video recordings, captured by video cameras (YI Outdoor Security Camera 1080p; YI Technology, Shanghai, China). To identify the focal cows in the videos, pink veterinary bandaging tape (Vetrap Bandaging Tape, 3M, London, ON, Canada) was attached to the hind legs of the focal cows before they entered the free-stall pens. The videos were recorded on a 32GB microSD card (SanDisk Ultra microSD UHS-I; Milpitas, California, United States of America) and replaced every 2 d with another microSD card. The cameras were set to record at 20 frames/s and were positioned approximately 5 m above the pens such that each pen was fully visible from one camera.

Feeding behavior of the focal cows was recorded using 10-min video scan sampling (as validated by Endres et al., 2005) from the moment of being grouped in the free-stall pen until d 7 after regrouping. For each scan, at 10-min intervals, a cow was considered feeding when her head was completely past the headlocks and over the feed. To calculate total time spent feeding (min/d), we multiplied the number of scans per day where the cow was feeding by 10 (Endres et al., 2005). To calculate daily idle standing time (min/d), time spent feeding (min/d) and lying (min/d) were subtracted from the total minutes of the day (1440 min/d) (Hart et al., 2013). Idle standing time included the time spent waiting to be and being milked, time spent drinking water, and other non-productive related activity.

The social behavior of the focal cows was observed and recorded from the video recordings for a continuous 4-h period per day for 3 d (for 12 h total) (von Keyserlingk et al., 2008; Talebi et al., 2014) after regrouping. Talebi et al. (2014) observed focal cows from video recording for 3 h after feed delivery; however, after plotting preliminary data, we observed continued high occurrence of behavioral interactions for both treatment groups continuing into the 4th hour after feed delivery; therefore, we decided to observe social behavior for 4 h after feed delivery. Focal cows were observed immediately after joining the new group on d 1 and were observed immediately after feed delivery time on d 2 and 3 after regrouping. Cows on the control treatment were fed daily at 0932 h ± 43:18 min, whereas cows on the CE treatment were fed daily at 0957 h ± 27:56 min. Using the ethogram in Table 2.2, the following behaviors were recorded: displacement from the feed bunk, displacement from the stall, displacement in the alley, head butting, threatening, head-to-head contact, allogrooming, self-grooming, use of the brush, and scratching against the pen (against fixtures in the pen). Additionally, behavior events were distinguished as either actor (e.g. the focal cow was the one initiating the event) or reactor (e.g. the focal cow was the one receiving the event). Allogrooming (as actor and reactor) and self-grooming behavior were recorded as a single event if it was uninterrupted or interrupted by less than 20 s and then resumed; if the interruption lasted more than 20 s before being continued it was considered as 2 grooming events (de Freslon et al., 2020). All behavior events recorded were summarized as the frequency of events/d (de Freslon et al., 2020). Furthermore, to determine the overall effect of treatments on social behavior, we summed some of the social behavior variables together, including total displacements (displacement from the stall, displacement from the feed bunk and displacement in the alley), aggression (head butting, threatening, head-to-head contact), and grooming (allogrooming, self-grooming, use of the brush, and scratching against the pen), both as actor and reactor. Further, the total displacements and aggression were summed together as the total competitive behavior (both as actor and reactor).

2.2.4 Feed sampling and analyses

Samples of each TMR (control and CE diets) were collected in duplicate 3x/wk. Orts (refusal) samples from each free-stall pen were taken 3x/wk. In addition, orts from focal cows were collected 3x/wk while the cows were in the tie-stall area. Focal cow orts in the tie-stall were weighed daily, and free-stall pen orts were weighed 3x/wk across the study period. Upon feed sampling, all samples were immediately frozen at −20°C until further analysis. Samples were thawed for 1 d prior to being dried. All samples were oven-dried at 55ºC for 48 h for DM analysis. Samples of TMR diets to be analyzed were ground by passing through a 1-mm screen (Model 4 Wiley Laboratory Mill, Thomas Scientific, Swedesboro, NJ). Ground samples were sent to A & L Laboratory Services Inc. (London, ON, Canada) for chemical analyses (Table 2.1) of ash (550°C; AOAC, 2000: method 942.05), ADF (AOAC, 2000: method 973.18), NDF with heat-stable α-amylase and sodium sulfite (AOAC, 2000: method 2002.04), CP (N × 6.25; AOAC 2000: method 990.03; Leco FP-628 Nitrogen Analyzer, Leco, St. Joseph, MI), starch (heat-stable amylase and amyloglucosidase; AOAC 2000: method 996.11), fat (using pet ether, AOAC 2000: method 920.39), lignin (using ADF residue and H2SO4), and minerals (using aquaregia digestion inductively coupled plasma atomic emission spectroscopy), and calculation of TDN and net energy (using NRC, 2001 equations).

2.2.5 Milk production and body weight and body condition score

Milk yield, at each milking, was measured and recorded for all cows throughout the study using the parlor milk weighing system and summarized on a daily basis (kg/d). This data was downloaded and backed up at minimum 3x/wk throughout the study. Body weight (BW) and body condition score (BCS) were recorded for all cows in the free-stalls, at each milking, across the study period using automated scale and automated BCS camera (DeLaval BCS; Delaval, Tumba, Sweden), as validated by Mullins et al. (2019), both placed on exit from the milking parlour. One value per cow per day was calculated by averaging across the afternoon and morning milkings.

2.2.6 Statistical analyses

All data were summarized on a daily basis with day beginning at 1000 h and ending at 0959 h the next day. All statistical analyses were conducted using SAS 9.4 software (SAS Institute Inc., 2013). For all analyses, outcomes were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Before analysis, all data were screened for normality and outliers using the UNIVARIATE procedure of SAS: milk yield, rumination time, lying time, idle standing time, and feeding time were normally distributed. Displacement from the feed bunk, displacement from the stall, displacement in the alley, head butting, threatening, head-to-head contact, allogrooming, self-grooming, use of the brush, scratching against the pen, displacement, aggression, grooming, and total competitive behavior (for both actor and reactor) were right-skewed (due to a large number of zero values) and transformed, to achieve normality, by taking the natural logarithm +1. In preliminary analyses of the behavioral responses of cows after regrouping, we detected a treatment by parity interaction; therefore, to better address our hypotheses, we proceeded to analyze the treatment effect for each response variable within parity group (primiparous and multiparous).

For milk yield, rumination time, and lying time, a baseline average of the 7 d before regrouping was generated, with d 1 representing the day that the focal cow was regrouped. These data were analyzed in repeated measures, mixed-effect linear regression models (using the MIXED procedure of SAS), treating day as a repeated measure. Models were specifically built to test whether the outcome variables after regrouping differed from their baseline average. Fixed effects in the models included treatment (control or CE), day (baseline average and d 1, 2, 3, 4, 5, 6, and 7 after regrouping), and the interaction between treatment and day. Cow was included as a random effect. The first-order autoregressive covariance structure was used for all models based on best fit, according to the lowest Bayesian information criterion values. Body weight, BCS, DIM, and 7-d average milk yield (of the focal cows) before entering the trial were all tested as covariates in the model. No interactions between these covariates and treatment were detected. To test the hypothesis that cows changed their behavior (i.e. lying and rumination) and milk yield following regrouping, differences between days after regrouping and their baseline average, within treatment, were compared.

Feeding time and idle standing time data were analyzed in repeated measures mixed-effect linear regression models (using the MIXED procedure of SAS), treating day as a repeated measure. Fixed effects in the models included treatment (control or CE), day (d 1, 2, 3, 4, 5, 6, and 7 after regrouping), and the interaction between treatment and day. Cow was included as a random effect. The first-order autoregressive covariance structure was used for both models based on best fit, according to the lowest Bayesian information criterion values. Body weight, BCS, and DIM were tested as covariates in the model. No interactions between these covariates and treatment were detected. When treatment by day interactions were detected, differences between treatments were compared by day after regrouping using the PDIFF option in the LSMEANS statement.

Additionally, to determine how the milk yield and rumination behavior of the focal cows compared with their pen mates after regrouping, we made a comparison of the difference in mean milk yield and rumination behavior between the focal cows and their pen mates. A mean milk yield and rumination time were generated by day for the 29 non-focal cows in each free-stall pen; this mean value was then subtracted from the values of the focal cows for the same day, resulting in new variables: difference of focal cow milk yield from the pen mean and difference of focal cow rumination time from the pen mean. These data were analyzed in repeated measures mixed-effect linear regression models (using the MIXED procedure of SAS), treating day as a repeated measure. Fixed effects in the models included treatment (control or CE), day (d 1, 2, 3, 4, 5, 6, and 7 after regrouping), and the interaction between treatment and day. Cow was included as a random effect. The first-order autoregressive covariance structure was used for all models based on best fit, according to the lowest Bayesian information criterion values. Body weight, BCS, and DIM were tested as covariates in the model. No interactions between these covariates and treatment were detected.

Social behavior variables analysed included displacement from the feed bunk (actor and reactor), displacement from the stall (actor and reactor), displacement in the alley (actor and reactor), head butting (actor and reactor), threatening (actor and reactor), head-to-head contact (actor and reactor), allogrooming (actor and reactor), self-grooming, use of the brush, and scratching against the pen), totaldisplacements (actor and reactor), total aggression (actor and reactor), total grooming (actor and reactor), and total competitive behavior (actor and reactor). These data were analyzed in repeated measures mixed-effect linear regression models (using the MIXED procedure of SAS), treating day as a repeated measure. Fixed effects in the models included treatment (control or CE), day (d 1, 2, and 3 after regrouping), and the interaction between treatment and day. Cow was included as a random effect. The first-order autoregressive covariance structure was used for all models based on best fit, according to the lowest Bayesian information criterion values. Body weight, BCS, and DIM were tested as covariates in the model. No interactions between these covariates and treatment were detected. When treatment by day interactions were detected, differences between treatments were compared by day after regrouping using the PDIFF option in the LSMEANS statement.

2.3 RESULTS

2.3.1 Dry matter intake

In the baseline period (7 d in tie-stall area prior to regrouping) primiparous control cows consumed 24.5 ± 0.5 kg/d of DM, whereas, primiparous CE cows consumed 24.8 ± 0.5 kg/d of DM. Multiparous cows that were fed the control diet consumed 30.2 ± 0.5 kg/d of DM and multiparous cows that were fed the CE diet consumed 29.5 ± 0.6 kg/d of DM in the baseline period. No differences were detected for DMI in the baseline period between the CE and control focal cows (for either primiparous or multiparous; $P \geq 0.1$). Across the whole study period, the DMI of the free-stall non-focal and focal control cows averaged 27.1 ± 2.3 kg/d, whereas, for the free-stall CE cows, it averaged 26.7 ± 2.7 kg/d.

2.3.2 Rumination behavior

Baseline rumination time of primiparous control and CE cows was 528.4 ± 21.8 and 485.0 ± 21.8 min/d, respectively (Table 2.3). Primiparous cows fed the control diet exhibited decreased rumination time on d 1 (P = 0.003) and d 2 (P = 0.02) after regrouping compared with their own baseline (Table 2.3), whereas, primiparous cows on the CE diet spent more time ruminating on d 2 (P = 0.03), d 3 (P = 0.002), d 4 (P = 0.02), and d 7 (P = 0.03), and tended to have greater rumination time on d 5 (P = 0.09) after regrouping compared with their baseline period average.

During the baseline period, multiparous control and CE cows spent 484.6 ± 25.0 and 490.7 ± 26.4 min/d ruminating, respectively (Table 2.3). Rumination time was increased from their baseline period average on d 5 (P = 0.009) and tended to increase on d 3 (P = 0.07) and 4 (P = 0.06) for multiparous control cows (Table 2.3); whereas multiparous cows fed the CE diet had greater rumination time on d 3, 4, and 5 (P < 0.05), and had a tendency for increased rumination time on d 2 (P = 0.07) and 7 (P = 0.06) when compared with their baseline period average (Table 2.3).

Primiparous control and CE non-focal free-stall cows ruminated 538.0 ± 21.1 min/d and 526.7 ± 21.2 min/d, respectively. Additionally, multiparous control and CE non-focal free-stall cows ruminated 548.4 ± 20.8 min/d and 515.7 ± 21.2 min/d, respectively. No treatment effect was detected in the difference of rumination time between focal primiparous cows and their primiparous pen mates in the 7 d after regrouping (Table 2.4). Multiparous CE cows had a greater mean difference of rumination time (+24.2 ± 14.4 min/d) compared with their multiparous pen mates than multiparous control cows (-32.6 ± 13.6 min/d) in the 7 d after regrouping (Table 2.4). No treatment x day interactions were detected for either the primiparous or multiparous models of difference in rumination time from their pen mates ($P \geq 0.1$).

2.3.3 Lying behavior

During the baseline period, primiparous control and CE cows spent an average of 807.6 ± 47.1 min/d and 776.6 ± 50.1 min/d lying down, respectively (Table 2.5). Compared with their baseline period, primiparous cows fed the control diet exhibited a reduction in lying time on d 1 (P = 0.002) and tended to have reduced lying time on d 2 (P = 0.06) and 3 (P = 0.06) following regrouping (Table 2.5). However, for primiparous cows fed the CE diet, no change was detected in lying time from their baseline period average for the 7 d following regrouping (Table 2.5).

Multiparous control and CE cows spent 757.9 ± 50.3 min/d and 774.2 ± 46.4 min/d, respectively, lying down during the baseline period (Table 2.5). For multiparous control cows there was no detected reduction in lying time on any of the 7 d after regrouping compared with their own baseline lying time (Table 2.5), whereas, multiparous CE cows exhibited a reduction in time spent lying on d 1, 2, 4, and 5 ($P < 0.05$), and tended to have reduced lying time on d 6 (P = 0.09).

2.3.4 Feeding time and idle standing time

Primiparous control focal cows spent 268.5 ± 20.5 min/d at the feed bunk, whereas, primiparous CE cows spent 282.4 ± 20.4 min/d in the 7 d after regrouping (Table 2.6). Furthermore, multiparous focal cows that were fed CE spent 284.78 ± 14.7 min/d at the feed bunk, whereas, multiparous focal cows that were fed the control diet spent 309.4 ± 14.8 min/d at the feed bunk. A treatment x day interaction was detected for primiparous focal cow feeding time (P = 0.04). Primiparous focal cows that were fed CE had greater feeding time on d 1 (P = 0.03) and tended to have greater feeding time on d 2 (P = 0.09; Table 2.6) compared with primiparous control focal cows after regrouping. Furthermore, primiparous cows that were fed the control diet spent more time at the feed bunk on d 7 (P = 0.04; Table 2.6; Figure 2.1) than primiparous CE cows after regrouping. No difference was detected for time spent at the feed bunk between multiparous control and CE focal cows in the 7 d after regrouping (Table 2.6).

Mean idle standing time for primiparous control and CE focal cows was 504.1 ± 49.6 min/d and 371.1 ± 38.8 min/d, respectively, whereas multiparous control focal cows spent 388.73 ± 43.4 min/d standing idle and multiparous CE focal cows spent 484.9 ± 38.0 min/d standing idle (Table 2.7). Primiparous CE cows spent less time standing idle (min/d) than primiparous control cows in the 7 d after regrouping (Table 2.7). Furthermore, no difference in idle standing time (min/d) was detected between multiparous CE and control cows in the 7 d after regrouping (Table 2.7).

2.3.5 Social behavior

The effect of treatment on the social behavior of primiparous focal cows is reported in (Table 2.8). As compared with the primiparous control cows, the primiparous CE focal cows displaced other cows less often in the alley and had less total competitive behavior as actor (displacement and aggression variables summed) (Table 2.8). Those cows also tended to head butt other cows less often in the pen, to initiate less head-to-head contact, to displace fewer cows from all areas of the pen (feed bunk, free stalls, and alley), and engage in less aggression across all 3 d after regrouping compared to the primiparous control cows (Table 2.8).

Primiparous focal cows that were fed the CE diet tended to be displaced more frequently from the feed bunk than primiparous focal cows that were fed the control diet after regrouping (Table 2.8). A treatment x day interaction was detected for displacements from the stall as reactor (Table 2.8), whereby primiparous CE focal cows tended be displaced from the stall more often on d 1 (P = 0.09) than primiparous control focal cows after regrouping (Figure 2.2). A treatment by day interaction was detected ($P \leq 0.1$; Table 2.8) for threatening (reactor), head butting (reactor), aggression (reactor), grooming, and total competitive behavior (reactor) primiparous focal cows; however, no differences between treatments within day were detected for any of these variables. We did not detect any differences between both treatments for allogrooming, self-grooming, use of the brush, and scratching against the pen after regrouping.

The effect of treatment on social behavior of multiparous focal cows is reported in Table 2.9. Multiparous CE focal cows were head butted more frequency than multiparous control focal cows in the 3 d after regrouping (P = 0.03). A tendency for treatment by day was detected for displacement from the free stall (Table 2.9). Multiparous focal cows that were fed the CE diet displaced fewer cows from the stall on d 1 after regrouping (P = 0.001; Figure 2.3) than multiparous focal cows that were fed the control diet.

2.3.6 Milk yield

In the baseline period, primiparous control and CE cows produced 30.5 ± 1.42 and 35.0 ± 1.42 kg/d of milk, respectively (Table 2.10). Primiparous cows fed the CE diet had greater milk yield than primiparous control cows across the 7 d after regrouping (Table 2.10). Additionally, primiparous control cows had decreased milk yield on d 1 after regrouping compared with their baseline average ($P < 0.001$; Table 2.10). Whereas, primiparous cows fed the CE diet had decreased milk yield on d 1 (P = 0.01) and 7 (P = 0.04) after regrouping as compared with their baseline period average.

During the baseline period, multiparous control and CE cows produced an average of 39.4 ± 1.87 and 40.8 ± 1.85 kg/d of milk, respectively (Table 2.10). For multiparous cows fed the control diet, no difference from the baseline period average in milk yield was detected in the 7 d after regrouping (Table 2.10), whereas multiparous cows fed the CE diet had decreased milk yield on d 1 ($P = 0.005$) and 2 ($P = 0.03$) compared with their baseline average.

Primiparous control and CE non-focal free-stall cows produced 29.8 ± 1.5 kg/d and 30.1 ± 1.4 kg/d of milk, respectively. Moreover, multiparous control and CE non-focal free-stalls cows produced 30.5 ± 2.1 kg/d and 30.6 ± 2.0 kg/d, respectively. Primiparous focal cows fed CE tended (Table 2.11) to have a greater difference in milk yield (+3.7 ± 1.6 kg/d) compared with their pen mates than primiparous control cows, which had a negative difference in milk yield (-0.5 ± 1.6 kg/d) compared with their pen mates in the 7 d after regrouping . No treatment effect was detected for the difference of milk yield between focal multiparous cows and their multiparous pen mates in the 7 d after regrouping (Table 2.11). No treatment x day interactions were detected for either the primiparous or multiparous models of difference in milk yield from pen mates ($P \geq 0.1$).

2.4 DISCUSSION

Regrouping of dairy cows has previously been demonstrated to have negative effects on cow behavior(von Keyserlingk et al., 2008; Mazer et al., 2020; Freslon et al., 2020) and milk production (Brakel and Leis, 1976; Hasegawa et al., 1997). Further, the effects of psychological stressors associated with regrouping of animals include reduced response to infection, increased fear response, and decreased milk production (Chen et al., 2015). In previous research, supplementation of CE has been demonstrated to have anxiolytic effects in rats (Faturi et al., 2010) and humans (Hekmatpou et al., 2017; Lehrner et al., 2000). To our knowledge, our work is the first to study the effects of a CE on the behavior and production of lactating cows after regrouping. Our objective was to determine if feeding a CE (derived from *Citrus sinensis*) would reduce the negative impact of regrouping of lactating dairy cows on behavior and milk production, particularly for primiparous cows. As hypothesized, supplementation of CE to primiparous cows led to a lesser change of behavior (lying time, rumination time), lesser competitive behavior, and greater milk production in the 7 d after introduction into a new social group. However, there was little detected effect of CE supplementation on the response of multiparous cows to regrouping.

Primiparous cows that were fed CE had greater feeding time on d 1 and tended to have greater feeding time on d 2 after regrouping as compared with the primiparous cows that were fed the control diet. Despite not being able to assess the change in feeding time upon regrouping of primiparous focal cows, the difference between the CE and control primiparous cows suggests that regrouping had a greater negative effect on the primiparous control cows feeding time compared with the CE cows. In previous work, Phillips and Rind (2001) demonstrated that both primiparous and multiparous cows that were regrouped into an assorted parity group had reduced grazing time after regrouping, due to greater aggressive interactions and competition.

In our study, time spent lying down also decreased drastically on the first day after regrouping and returned to baseline after 3 d for the primiparous control focal cows. This is consistent with previous research that also detected a decrease in lying time on d 1 after regrouping (von Keyserlingk et al., 2008). Moreover, von Keyserlingk et al. (2008) suggested that focal cows were more unwilling to displace their pen mates to gain access to a preferred stall, thus, spending less time lying down following regrouping. Alternatively, in this current study, lying time was sustained in the 7 d after regrouping for the primiparous CE cows, again indicating that CE reduced the negative effect of regrouping. The sustained lying and greater feeding time immediately after grouping of the primiparous CE focal cows in our study may have contributed to their lesser idle standing time. Overall, this indicates that primiparous CE cows used their time more efficiently after regrouping as compared with the control primiparous cows.

Inconsistent results have previously been reported for the effect of regrouping cows on rumination time. Hasegawa et al. (1997) reported no change in rumination time after cows were regrouped. However, in that study the investigations of rumination time of the cows started 2 d after regrouping and, therefore, a drop in rumination may have been missed. Alternatively, in a study with dry cows fed with Insentec feed bins, rumination time was decreased on d 1 after regrouping (Schirmann et al., 2011). Those researchers suggested that the decrease in rumination time after regrouping was associated with decreased DMI following regrouping. We can theorise that in our study the sustained rumination time of the primiparous CE cows following regrouping was associated with greater feeding on d 1 and 2 and the sustained lying time (Schirmann et al., 2012), and potentially a change in DMI given its association with both greater feeding and rumination time (Johnston and DeVries, 2018). However, we were not able to measure individual DMI of the socially grouped focal cows, thus, challenging the ability to predict if the rumination time in our study was driven primarily by DMI (Schirmann et al., 2012).

von Keyserlingk et al. (2008) reported that focal cows were displaced more regularly at the feed alley by their pen mates in the 3 d following regrouping as compared with their baseline value. Schirmann et al. (2011) also reported that focal cows displaced more cows at the feed bin on d 1 after regrouping, likely due to greater competition there. Although our study was not designed to assess any change in aggressive behavior from the baseline, we did detect differences in aggressive behaviors between the CE and control primiparous cows. Researchers have demonstrated that rats and pigs may react to social stressors showing more aggressive behavior (Koolhaas et al., 1999; Bolhuis et al., 2005). However, Nogues et al. (2020) reported in their study that some heifers avoided engaging in aggressive interactions, whereas, other heifers engaged in more aggressive interactions after regrouping. Interestingly, those heifers less willing to engage spent less time feeding and resting than the heifers that were more willing to engage in aggressive interactions to access important resources (i.e. feed bunk, lying stalls, waterers). These findings suggest that heifers undergoing psycho-social stress may vary in their aggressive behavior after regrouping due to their individual characteristics. In the current study, primiparous cows that were fed CE tended to be displaced more frequently from the feed bunk and from the free stalls on d 1 after regrouping as compared with the primiparous control cows, likely reflecting the fact that the primiparous CE cows spent more time eating at the feed bunk and lying in the free stalls. Thus, those cows would have been more susceptible to be displaced at those places than the primiparous control cows, who had greater idle standing time (away from the stalls and bunk) after regrouping. Potentially related to that greater idle standing time, those primiparous control cows displaced other cows more often in the alley, had more total social behaviors observed as actor (displacements and aggression variables summed), and tended to head butt other cows more often in the pen, to initiate more head-to-head contact, to displace more cows in the pen, and engage in more aggression than primiparous CE focal cows across all 3 d after regrouping. Greater psycho-social stress following regrouping may be a potential explanation for the greater frequency of these aggressive behaviors initiated by the primiparous control focal cows, as compared with the primiparous CE cows. Psycho-social stress can be defined as social events (regrouping, weaning, restraint) that trigger a psychological stress response, disturbing the homeostatic state of the animal (Chen et al., 2015). The present results support our hypothesis that feeding CE to cows would mitigate the psycho-social stress effects of moving cows into a new social group. In previous research it has been demonstrated that CE will mitigate responses to psycho-social stress related to dietary transitions and anxiolytic situations (Faturi et al., 2010; Clouard et al., 2012; Val-Laillet et al., 2016).

Increased aggressive behavior of cows after regrouping may lead to more psycho-social stress, which can decrease milk yield (Chen et al., 2015). Similar to other studies (Brakel and Leis, 1976; Hasegawa et al., 1997; von Keyserlingk et al., 2008), both primiparous CE and control focal cows experienced a reduction in milk yield on the first day following regrouping. However, despite controlling for pre-trial milk yield, the primiparous CE cows had greater milk yield across the 7 d after regrouping as compared with the primiparous cows fed the control diet. The greater milk yield in the primiparous CE cows as compared with primiparous control cow after regrouping may have been due to those cows having overall greater feeding time immediately after grouping, less idle standing time and initiating lesser aggressive interaction than primiparous control cows after regrouping. Further, those cows also had sustained rumination and lying time compared with their baseline as compared to the primiparous control cows after regrouping. Ensuring cows have sufficient time to devote to these behaviors is important for the maintenance of milk production (Johnston and DeVries, 2018; Kaufman et al., 2018). We theorize that primiparous cows supplemented with CE have reduced anxiety, therefore reducing aggressive behavior and allowing those cows to spend more time at productive behaviors (i.e. feeding, lying, ruminating, etc.) following regrouping.

No difference in feeding time was detected between multiparous CE and control focal cows after regrouping. Further, rumination time was sustained on d 1 and increased on some days for both multiparous CE and control focal cows after regrouping. The multiparous CE focal cows had more days with increased rumination time after regrouping as compared with their baseline than multiparous control cows, suggesting that CE may have also had some positive effect on the multiparous cows. However, the multiparous CE cows also had decreased time spent lying on most days after regrouping, whereas, the multiparous control cows sustained their lying time across the 7 d of regrouping. These results are difficult to interpret given that rumination and lying time are often positively correlated (Schirmann et al., 2012). CE supplementation had little effect on the social behavior of the multiparous cows, with the multiparous CE focal cows being head butted more often than multiparous control cows across the 3 d following regrouping and the multiparous control cows displacing more cows from the stall on d 1. Furthermore, the multiparous CE cows had reduced milk yield on d 1 and 2 after regrouping compared with their baseline. However, this result does not correspond with other measures of psycho-social stress (e.g. rumination, lying time, idle standing, feeding behavior, and social behavior). Therefore, there is not sufficient evidence to support that the reduced milk yield detected in multiparous CE cows was due to the social-related affects that came with moving them from individual to group housing and not due to other unknown effects.

We hypothesise that the lesser CE effect observed for the multiparous focal cows may be due to the lesser challenging effect that regrouping has on multiparous cows. Overall, the primiparous control cows experienced a reduction in rumination time, lying time, and milk yield compared with their baseline after regrouping. In contrast, the multiparous control cows did not experience as substantial decrease in rumination time, lying time, and milk yield after regrouping as compared with their baseline. This is not entirely surprising, given that primiparous cows were reported to have less competitive success at the feed bunk than multiparous cows (Huzzey et al., 2012), and to have greater fecal cortisol when regrouped individually than multiparous cows regrouped individually (Mazer et al., 2020). Thus, these studies may indicate that primiparous cows are more susceptible to psycho-social stress than multiparous cows.

2.5 CONCLUSIONS

Feeding CE to primiparous cows mitigated the negative effect of regrouping on behavior and milk production. Specifically, primiparous cows fed CE had greater milk yield as compared with primiparous cows fed the control diet across the 7 d following regrouping. Additionally, primiparous CE cows had a lesser change in time spent lying and ruminating compared with their baseline after regrouping. Primiparous cows fed CE had greater feeding time immediately after regrouping as compared to primiparous control cows, and as a result of sustained lying time, the primiparous CE cows displayed less idle standing time. Overall, the primiparous control cows initiated a greater number of competitive interactions, whereas the primiparous CE cows received more aggressive interactions after regrouping. Although feeding CE to primiparous cows reduced the negative effects of regrouping, CE supplementation had little effect on the response of multiparous cows to regrouping, suggesting that the effect of regrouping may be greater for primiparous cows compared to multiparous cows.

2.6 ACKNOWLEDGEMNTS

Thank you to the staff of the University of Guelph, Elora Research Station – Ontario Dairy Research Centre (Elora, ON, Canada), and especially to Jeff McFarlane and Laura Wright for all their assistance facilitating this research project. Special thanks to Kaitlyn Dancy, Brooke McNeil, Meagan King, and Bruna Mion of the University of Guelph (Guelph, ON, Canada) for their assistance with data collection. This project was financially supported by Phodé (Terssac, France) and through a Natural Sciences and Engineering Research Council of Canada (NSERC; Ottawa, ON, Canada) Discovery Grant, as well as received support from the Ontario Agri-Food Innovation Alliance Research Program of the University of Guelph and the Ontario Ministry of Agriculture, Food, and Rural Affairs (Guelph, ON, Canada). Further, project equipment was supported by contributions from the Canadian Foundation for Innovation (CFI; Ottawa, Ontario, Canada) and the Ontario Research Fund (Toronto, ON, Canada).

Table 2.1. Ingredient and chemical composition (mean ± SD) of the lactating cow diet[1]

Composition	CE	Control
Ingredient, % of DM		
Corn Silage[2]	29.3	
Wheat Straw[3]	1.8	
Alfalfa Haylage[4]	29.5	
High Moisture Corn[5]	25.3	
Lactating Cow Supplement[6]	14.1	
Chemical Composition[7]		
DM, %	48.1 ± 1.40	48.2 ± 1.76
CP, % of DM	14.7 ± 0.67	14.7 ± 1.01
ADF, % of DM	20.4 ± 0.73	20.0 ± 0.98
NDF, % of DM	29.3 ± 0.86	29.3 ± 1.40
TDN, % of DM	73.0 ± 0.57	73.3 ± 0.76
Starch, % of DM	28.4 ± 1.92	29.7 ± 2.04
Sugar, % of DM	3.5 ± 0.58	3.3 ± 0.24
NFC, % of DM	44.5 ± 0.60	44.5 ± 1.61
Ca, % of DM	0.9 ± 0.05	0.9 ± 0.05
P, % of DM	0.4 ± 0.04	0.4 ± 0.05
K, % of DM	1.4 ± 0.11	1.4 ± 0.14
Na, % of DM	0.4 ± 0.03	0.4 ± 0.04
Mg, % of DM	0.4 ± 0.02	0.4 ± 0.03
NE_L, Mcal/kg of DM)	1.7 ± 0.01	1.7 ± 0.02

[1]CE = lactating cow diet with control TMR with 4 g/d of citrus extract; Control = lactating cow diet.

[2]Corn silage had a DM of 33.4%.

[3]Straw had a DM of 89.2%.

[4]Alfalfa haylage had a DM of 32.8.

[5]High moisture corn had a DM of 74.0 ± 2.6% and chemical composition (DM basis) 8.0 ± 1.3% CP, 2.8 ± 0.4% ADF, and 8.5 ± 0.8% NDF.

[6]Supplied by Floradale Feed Mill Ltd (Floradale, Ontario, Canada) including ingredients (as is); 40% Bypass Soybean meal, 29% soybean meal, 7% Canola meal, 5.6% Wheat shorts, 3% limestone calcium carbonate, 3.1% sodium sesquicarbonate, 3% fine salt, 2.8% monocalcium phosphate, 1.7% magnesium oxide, 1.4% Diamond V Yeast XP, 1% Tallow, 0.75% Integral, 0.7% Inorganic lactating mix, 0.5% Metasmart, 0.3% DCAD+, 0.04 Sulphur, and 0.05% Rumensin,. Lactating cow supplement had a DM of 89.3%.

[7]Values were obtained from chemical analysis of TMR samples. NE_L was calculated based on NRC (2001) equations.

Table 2.2. An ethogram of behaviors recorded from video observations

Behavior	Description
Event (actor[1] and reactor[2])	
Displacement from the feed bunk[3]	A cow (actor) uses her head, shoulders, or flank to aggressively contact another cow (reactor), causing her to retreat (withdrawing her head) from the feed bunk
Displacement from the stall[4]	A cow (actor) uses her head, shoulders, or flank to aggressively contact with another cow (reactor), causing her to completely retreat from the stall
Displacement in the alley[5]	A cow (actor) uses her head, shoulders, or flank to aggressively contact with another cow (reactor), causing her to retreat to another place
Head butting[6]	When a cow (actor) uses her head to make contact with another cow (reactor)
Threatening[7]	When a cow (actor) chases or approaches another cow (reactor), causing this cow to withdraw from her current location
Head-to-head contact[8]	When a cow (actor) initiates contact using the front of her head in the direction of another cow and the other cow (reactor) retributes by also contacting the actor with the front of her head
Allogrooming[9]	When a cow (actor) initiates a grooming event on another cow (reactor)
Event (actor[1])	
Self-grooming[10]	When a focal cow initiates a grooming event on herself
Use of the brush[11]	When a focal cow makes repetitive contact with their head or body against a brush
Scratching[12]	When a focal cow makes repetitive contact with their head or body against a fixture of the pen (e.g. a bar or a gate)
Summed behaviors (actor[1])	
Displacement	Sum of displacements from the feed bunk, displacements from the stall, and displacements in the alley
Aggression	Sum of threatening, head butting and head-to-head events
Grooming	Sum of allogrooming, self-grooming, use of the brush, and scratching
Summed behaviors (reactor[2])	
Displacement	Sum of times that the focal cows was displaced from the feed bunk, stalls, and in the alley
Aggression	Sum of times that the focal cows received an aggression (threatening, head butting and head-to-head) event
Total summed behaviors	
Total competitive behavior actor	Sum of all actor displacements and aggression
Total competitive behavior reactor	Sum of all reactor displacements and aggression

[1]Actor = Individual focal cow initiates the behavior event.

[2]Reactor = Individual focal cow that receives the behavior event.
[3]Displacement from the feed bunk (DeVries et al., 2010).
[4]Displacement from the stall (Fregonesi et al., 2007).
[5]Displacement from the alley (Val-Laillet et al., 2008).
[6] Head butting (Boyle et al., 2012).
[7]Threatening (Boyle et al., 2012).
[8]Head-to-head contact (Boyle et al., 2012).
[9]Allogrooming (von Keyserlingk et al., 2008; Boyle et al., 2012).
[10]Self-grooming (von Keyserlingk et al., 2008; Boyle et al., 2012).
[11]Use of the brush (Fregonesi et al., 2007).
[12]Scratching (DeVries et al., 2007).

Table 2.3. Effect of treatment on the rumination time (min/d) of primiparous and multiparous Holstein dairy cows after regrouping

	Baseline[1]	Day after regrouping							SEM	*P*-value[2]
		1	2	3	4	5	6	7		
Primiparous										
Control[3]	528.4	459.1*	477.6*	524.3	498.9	500.6	511.3	530.1	20.9	0.40
Citrus extract[4]	485.9	484.3	533.3*	556.3*	536.3*	523.3†	521.6	535.3*	20.9	
Multiparous										
Control[3]	484.6	517.7	524.8	540.0†	541.8†	564.0*	526.4	525.1	24.0	0.60
Citrus extract[4]	490.7	499.7	549.1†	565.3*	571.1*	556.6*	536.9	554.0†	25.4	

[1]Baseline = Average of 7-d prior to regrouping.

[2] P-value for overall effect of treatment.

[3]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).

[4]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).

*indicates difference from Baseline value within treatment at $P \leq 0.05$.

†indicates tendency for difference from Baseline value within treatment at $0.05 < P \leq 0.10$.

Table 2.4. Effect of treatment on the difference of rumination time (min/d) between primiparous and multiparous Holstein focal cows and their non-focal free-stall Holstein cow pen mates in day after regrouping[1]

	Day after regrouping							SEM	*P*-value[2]
	1	2	3	4	5	6	7		
Primiparous									
Control[3]	-149.9	-66.4	-87.7	-28.0	-57.8	-12.8	-46.7	32.7	0.11
Citrus extract[4]	-170.5	0.4	37.5	-0.3	-16.4	25.4	-26.9	30.8	
Multiparous									
Control[3]	-167.7	-18.2	-4.0	9.2	-14.90	-10.9	-21.5	24.6	0.005
Citrus extract[4]	-122.1	25.4	48.1	69.9	52.5	25.2	70.6	25.9	

[1]Mean rumination per day for the 29 non-focal cows in each free-stall pen were subtracted from the values of the focal cows for the same day.
[2]P-value for overall effect of treatment.
[3]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[4]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).

Table 2.5. Effect of treatment on the lying time (min/d) of primiparous and multiparous Holstein dairy cows after regrouping

	Baseline[1]	Day after regrouping							SEM	*P*-value[2]
		1	2	3	4	5	6	7		
Primiparous										
Control[3]	807.6	593.9*	672.1†	673.4†	783.1	719.2	698.6	693.0	62.4	0.22
Citrus extract[4]	776.6	780.0	728.4	746.7	805.7	786.3	784.7	711.3	50.5	
Multiparous										
Control[3]	757.9	720.6	751.1	706.0	759.8	698.54	676.1	805.5	47.8	0.18
Citrus extract[4]	774.2	660.4*	660.7*	683.1	656.1*	661.33*	677.4†	703.1	42.8	

[1]Baseline = Average of 7-d prior to regrouping.
[2]P-value for overall effect of treatment.
[3]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[4]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).
*indicates difference from Baseline value within treatment at $P \leq 0.05$.
†indicates tendency for difference from Baseline value within treatment at $0.05 < P \leq 0.10$.

Table 2.6. Effect of treatment on the feeding time (min/d) of primiparous and multiparous Holstein dairy cows after regrouping

	Day after regrouping							SEM	*P*-value[1]
	1	2	3	4	5	6	7		
Primiparous									
Control[2]	212.7	221.7	277.3	281.4	300.3	253.4	332.4	29.1	0.63
Citrus extract[3]	301.4	290.0	305.7	284.3	295.7	261.8	238.0	28.8	
Multiparous									
Control[2]	280.3	311.7	302.1	321.5	339.2	287.4	323.8	20.4	0.24
Citrus extract[3]	263.0	275.4	291.4	288.6	285.9	299.4	289.7	20.0	

[1]P-value for overall effect of treatment.
[2]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[3]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).

Table 2.7.. Effect of treatment on the idle standing time (min/d) of primiparous and multiparous Holstein dairy cows after regrouping

	Day after regrouping							SEM	*P*-value[1]
	1	2	3	4	5	6	7		
Primiparous									
Control[2]	685.25	549.00	534.58	386.75	447.69	488.84	436.70	81.10	0.04
Citrus extract[3]	352.83	415.17	383.67	329.67	334.00	367.38	414.76	60.61	
Multiparous									
Control[2]	470.66	362.82	367.48	358.08	418.48	411.92	331.68	56.04	0.11
Citrus extract[3]	537.24	528.14	457.46	477.52	484.98	462.75	446.07	47.79	

[1]P-value for overall effect of treatment.
[2]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[3]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).

Table 2.8. Effect of treatment on the behavior (#/d; natural log + 1)[1] of primiparous Holstein dairy cows after regrouping (mean ± SE)

	Treatment (T)		P-Value[2]		
Variable	CON[3]	CE[4]	Treatment	Day	T x D
Actor					
Displacement from the feed bunk	0.86 ± 0.24	0.64 ± 0.23	0.51	0.42	0.93
Displacement from the free stall	0.32 ± 0.14	0.28 ± 0.14	0.86	0.64	0.62
Displacement in the alley	1.12 ± 0.17	0.54 ± 0.16	0.02	0.13	0.43
Head butting	1.39 ± 0.23	0.75 ± 0.22	0.06	0.002	0.16
Threatening	0.65 ± 0.25	0.24 ± 0.25	0.27	0.006	0.39
Head-to-head contact	1.14 ± 0.22	0.51 ± 0.21	0.06	0.001	0.92
Allogrooming	0.33 ± 0.12	0.44 ± 0.11	0.47	0.64	0.68
Displacement[5]	1.62 ± 0.28	0.89 ± 0.27	0.08	0.13	0.94
Aggression[6]	1.92 ± 0.33	0.96 ± 0.32	0.06	<0.001	0.73
Total competitive behavior[7]	2.34 ± 0.35	1.32 ± 0.34	0.03	0.003	0.94
Reactor					
Displacement from the feed bunk	1.78 ± 0.26	2.42 ± 0.25	0.096	0.47	0.16
Displacement from the free stall	0.45 ± 0.17	0.51 ± 0.17	0.82	0.35	0.02
Displacement in the alley	1.58 ± 0.23	1.14 ± 0.22	0.19	0.01	0.66
Head butting	2.12 ± 0.34	1.87 ± 0.33	0.60	0.002	0.05
Threatening	2.06 ± 0.28	2.05 ± 0.28	0.97	<0.001	0.008
Head-to-head contact	1.09 ± 0.21	0.96 ± 0.20	0.68	0.01	0.62
Allogrooming	0.50 ± 0.15	0.54 ± 0.15	0.85	0.54	0.42
Displacement[8]	2.45 ± 0.23	2.73 ± 0.22	0.38	0.04	0.22
Aggression[9]	2.84 ± 0.29	2.80 ± 0.29	0.90	<0.001	0.002
Total competitive behavior[10]	3.30 ± 0.27	3.47 ± 0.27	0.67	0.002	0.007
Scratching against the pen	0.65 ± 0.23	0.70 ± 0.23	0.90	0.51	0.51
Self-grooming	1.74 ± 0.27	1.71 ± 0.26	0.93	0.22	0.59
Brush	0.67 ± 0.21	0.86 ± 0.20	0.52	0.007	0.51
Grooming[11]	2.28 ± 0.23	2.41 ± 0.23	0.70	0.92	0.10

[1]All behavior variables (individual and summed) were log-transformed (natural log + 1), given that they did not meet the assumption of normality.

[2]P-values are provided for the effects of Treatment (T), Day (D), and T × D.
[3]CON = control TMR (control diet; n=7).
[4]CE = control TMR with 4 g/d of citrus extract (CE diet; n=7).
[5]Sum of displacement variables (feed bunk, free stalls, alley) as actor.
[6]Sum of threatening, head butting, and head-to-head as actor.
[7]Total competitive behavior variable is all displacements and aggression summed as actor.
[8]Sum of displacement variables (feed bunk, free stalls, alley) as reactor.
[9]Sum of threatening, head butting, and head-to-head as reactor.
[10]Total competitive behavior variable is all displacements and aggression summed as reactor.
[11]Sum of allogrooming (actor and reactor), self-grooming, scratching agains the pen, and brush.

Table 2.9. Effect of treatment on the behavior (#/d; natural log + 1)[1] of multiparous Holstein dairy cows after regrouping (mean ± SE)

	Treatment (T)		P-Value[2]		
Variable	CON[3]	CE[4]	Treatment	Day	T x D
Actor					
Displacement from the feed bunk	1.21 ± 0.31	1.38 ± 0.31	0.70	0.60	0.97
Displacement from the free stall	0.33 ± 0.08	0.18 ± 0.08	0.23	0.02	0.08
Displacement in the alley	0.96 ± 0.16	1.05 ± 0.17	0.69	<0.001	0.36
Head butting	1.28 ± 0.23	1.66 ± 0.23	0.26	<0.001	0.23
Threatening	0.72 ± 0.18	0.92 ± 0.18	0.45	0.002	0.99
Head-to-head contact	0.90 ± 0.16	0.78 ± 0.16	0.62	<0.001	0.42
Allogrooming	0.42 ± 0.13	0.19 ± 0.13	0.23	0.42	0.91
Displacement[5]	1.78 ± 0.26	1.90 ± 0.27	0.74	0.01	0.85
Aggression[6]	1.84 ± 0.23	2.08 ± 0.24	0.48	<0.001	0.19
Total competitive behavior[7]	2.49 ± 0.25	2.65 ± 0.26	0.66	<0.001	0.49
Reactor					
Displacement from the feed bunk	1.63 ± 0.30	1.96 ± 0.30	0.44	0.36	0.18
Displacement from the free stall	0.30 ± 0.10	0.48 ± 0.11	0.23	0.01	0.28
Displacement in the alley	1.02 ± 0.22	1.28 ± 0.22	0.41	0.01	0.60
Head butting	1.48 ± 0.21	2.14 ± 0.21	0.03	<0.001	0.84
Threatening	1.25 ± 0.22	1.36 ± 0.23	0.71	<0.001	0.64
Head-to-head contact	0.84 ± 0.21	0.82 ± 0.21	0.94	<0.001	0.15
Allogrooming	0.42 ± 0.17	0.44 ± 0.17	0.94	0.18	0.54
Displacement[8]	1.98 ± 0.30	2.38 ± 0.30	0.35	0.002	0.27
Aggression[9]	2.19 ± 0.20	2.55 ± 0.21	0.22	<0.001	0.55
Total competitive behavior[10]	2.78 ± 0.23	3.16 ± 0.23	0.26	<0.001	0.52
Scratching against the pen	0.51 ± 0.16	0.59 ± 0.17	0.73	0.67	0.36
Self-grooming	1.30 ± 0.20	1.59 ± 0.20	0.30	0.74	0.75
Brush	0.52 ± 0.10	0.68 ± 0.10	0.27	0.02	0.88
Grooming[11]	1.93 ± 0.18	2.08 ± 0.19	0.58	0.50	0.71

[1]All behavior variables (individual and summed) were log-transformed (natural log + 1), given that they did not meet the assumption of normality.
[2]P-values are provided for the effects of Treatment (T), Day (D), and T × D.
[3]CON = control TMR (control diet; n=9).
[4]CE = control TMR with 4 g/d of citrus extract (CE diet; n=9).
[5]Sum of displacement variables (feed bunk, free stalls, alley) as actor.
[6]Sum of threatening, head butting, and head-to-head as actor.
[7]Total competitive behavior variable is all displacements and aggression summed as actor.
[8]Sum of displacement variables (feed bunk, free stalls, alley) as reactor.
[9]Sum of threatening, head butting, and head-to-head summed as reactor.
[10]Total competitive behavior variable is all displacements and aggression summed as reactor.
[11]Sum of allogrooming (actor and reactor), self-grooming, scratching agains the pen, and brush.

Table 2.10. Effect of treatment on milk yield (kg/d) of primiparous and multiparous Holstein dairy cows after regrouping

	Baseline[1]	Day after regrouping							SEM	*P*-value[2]
		1	2	3	4	5	6	7		
Primiparous										
Control[3]	30.5	28.1*	29.1	29.8	29.3	30.3	30.1	30.1	1.4	0.03
Citrus extract[4]	35.0	32.8*	33.8	34.6	33.5	33.5	34.0	32.9*	1.4	
Multiparous										
Control[3]	39.4	38.2	38.8	39.4	40.5	40.5	41.0	40.3	1.9	0.94
Citrus extract[4]	40.8	38.2*	38.0*	40.0	39.3	40.0	39.2	41.1	1.9	

[1]Baseline = Average of 7-d prior to regrouping.
[2]P-value for overall effect of treatment.
[3]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[4]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).
*indicates difference from Baseline value within treatment at $P \leq 0.05$.
†indicates tendency for difference from Baseline value within treatment at $0.05 < P \leq 0.10$.

Table 2.11. Effects of treatment on the difference of milk yield (kg/d) between primiparous and multiparous Holstein focal cows and their non-focal free-stall Holstein cow pen mates on the day after regrouping[1]

	Day after regrouping							SEM	*P*-value[2]
	1	2	3	4	5	6	7		
Primiparous									
Control[3]	-3.3	-1.2	-0.3	-0.7	0.9	0.6	0.3	1.8	0.06
Citrus extract[4]	3.1	3.8	4.7	3.2	3.4	4.5	3.4	1.8	
Multiparous									
Control[3]	7.8	8.3	9.3	9.9	10.3	11.2	10.2	2.1	0.86
Citrus extract[4]	8.0	7.7	9.8	8.9	9.7	9.0	10.5	2.1	

[1]Mean milk yield per day for the 29 non-focal cows in each free-stall pen were subtracted from the values of the focal cows for the same day.
[2]P-value for overall effect of treatment.
[3]Control = control TMR (control diet; n=16: primiparous = 7; multiparous = 9).
[4]Citrus extract = control TMR with 4 g/d of citrus extract (CE diet; n=16: primiparous = 7; multiparous = 9).

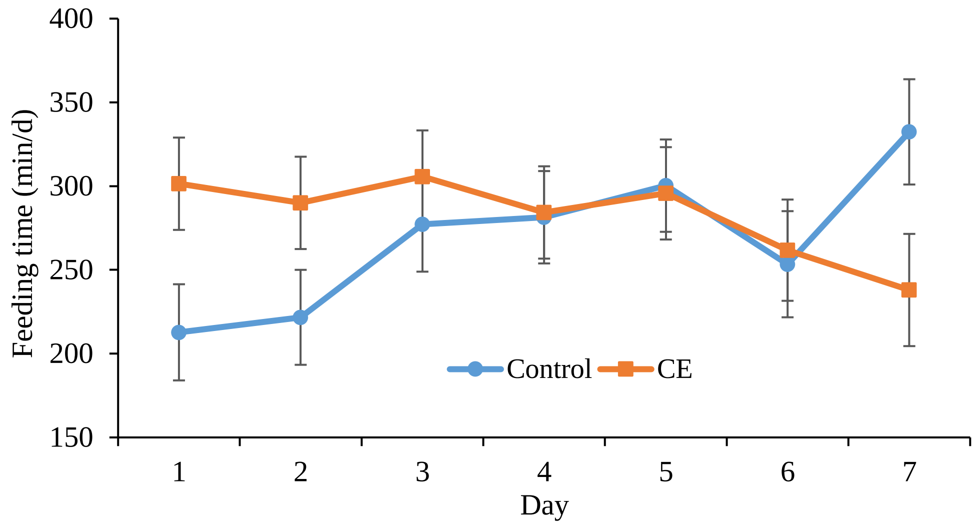

Figure 2.1. Effect of treatment on feeding time (mind/d) (mean ± SE) of primiparous lactating cows: (1) control TMR (control diet; n=7) or (2) control TMR with 4 g/d of citrus extract (CE diet; n = 7). Day = day following regrouping.

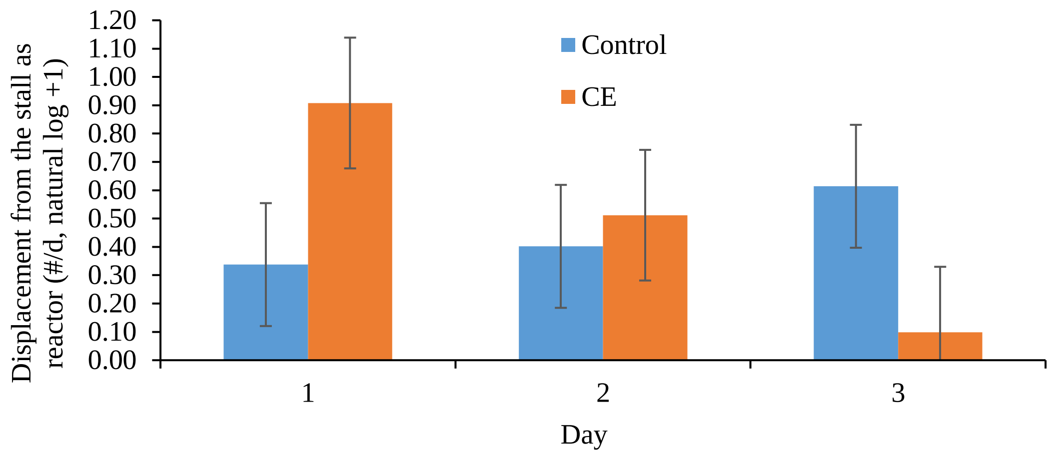

Figure 2.2. Effect of treatment on displacement (as reactor) from the stall (#/d, natural log +1; mean ± SE) of primiparous lactating cows: (1) control TMR (control diet; n=7) or (2) control TMR with 4 g/d of citrus extract (CE diet; n = 7). Day = day following regrouping.

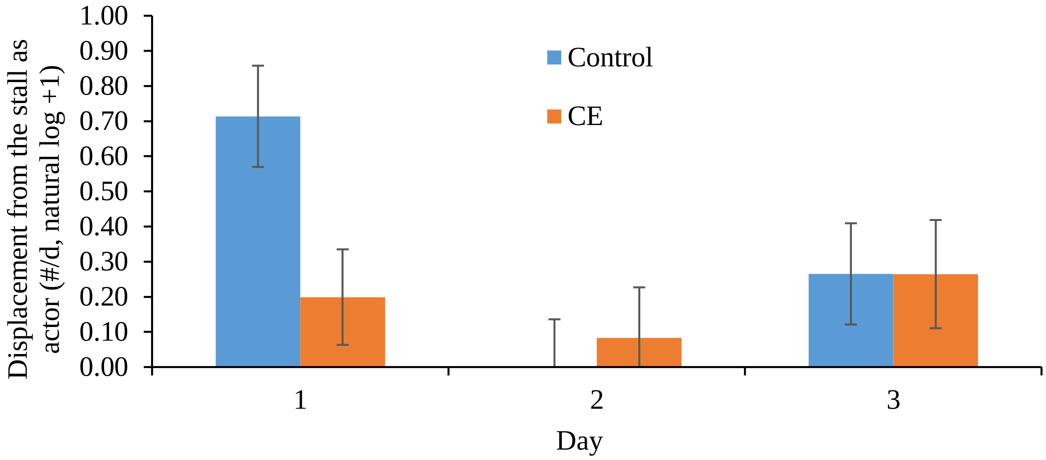

Figure 2.3. Effect of treatment on displacement (as actor) from the stall log transformed (#/d, natural log +1; mean ± SE) of multiparous lactating cows: (1) control TMR (control diet; n=9) or (2) control TMR with 4 g/d of citrus extract (CE diet; n = 9). Day = day following regrouping.

CHAPTER 3: GENERAL DISCUSSION

3.1 Major findings

Grouping and regrouping of dairy cows is an everyday activity on commercial dairy farms. The negative effects of regrouping of cows include decreased milk yield, lying time, rumination, and increased agonistic behavior. Cows may experience regrouping events up to 4 to 5 times per lactation (Grant and Albright, 2001). There are a few recommendations on regrouping in the Canadian Code of Practice for the Care and Handling of Dairy Cattle (NFACC, 2009). However, that document does not provide sufficient information on the regrouping topic, and their recommendations only include moving animals with established bonds together, moving animals in groups as opposed to individually, moving them at feeding time, and to avoid overstocking (NFACC, 2009). While those management practices have been demonstrated to reduce some of the negative effects of regrouping, more work is needed to fully mitigate these effects to maintain cow production and welfare. The use of sensory additives has been demonstrated in other species to reduce responses in stressful situations. For example, citrus extract (CE; derived from *Citrus sinensis)* has been reported to reduce anxiety and instill calmness in individuals experiencing stressful situations (Lehrner et al., 2005; Faturi et al., 2010), therefore stimulating feed appetite and acceptance in piglets (Astell et al., 2013; Clouard and Val-Laillet, 2014). However, the effects of CE on mitigating psycho-social stress of dairy cows have not been previously studied. Thus, my thesis was aimed at studying the effects of supplementing CE on mitigating the negative effects of moving dairy cows into a new social group on their behavior and production.

In my thesis research, the changes in behavior (lying time and rumination time) and milk yield after regrouping reinforce the known negative effects of this management practice. Citrus extract supplementation may have reduced those negative effects, particularly in primiparous cows as compared to multiparous cows. Overall, the primiparous cows fed the CE diet spent more time at the feed bunk in the first couple of days following regrouping as compared to those fed the control diet. Additionally, those cows sustained their lying time and rumination time in the days after regrouping compared with their baseline, whereas the control primiparous cows experienced decreases in time spent on those behaviors following movement into a new social group. Comparable to the primiparous control cow findings, researchers have previously reported that cows had decreased lying time and rumination time after regrouping, likely due to increased aggressive interactions leading to decreased DMI (von Keyserlingk et al. 2008; Schirmann et al 2011). In fact, the primiparous cows fed the CE diet demonstrated greater rumination time in the days following the first day after regrouping compared with their baseline. Greater feeding and lying time of the primiparous cows fed the CE diet likely contributed to the finding that they spent less time standing idle across the 7 d following regrouping as compared to the control primiparous cows. The feeding and lying time of the primiparous control cows is consistent with previous research where a decrease in those behaviors after regrouping was reported (Phillips et al., 2001; von Keyserlingk et al. 2008). It is interesting to note that the primiparous CE cows were more often the recipient of displacements from the feed bunk and free stalls after regrouping than the primiparous control cows, likely due to the fact that those CE cows were spending more time at those resources (feed bunk and lying stalls). On the other hand, the primiparous control cows initiated more aggressive interactions throughout other locations in the pen after regrouping than did the primiparous CE cows. Similarly, researchers have reported related findings with cows increasing overall agonistic behavior after being regrouped (von Keyserlingk et al. 2008; Schrimann et al., 2011). This coincides with the increased idle standing time (i.e. away from feeding and lying areas) of those control primiparous cows. Overall, these results suggest that the control primiparous cows were more socially disturbed after regrouping compared to the CE cows. Finally, the primiparous CE focal cows had greater milk yield than primiparous control cows across the 7 d following regrouping. These results for the primiparous cows collectively suggest that the CE supplementation mitigated the negative effects of regrouping, allowing those cows to dedicate more time to productive behaviors (eating, lying, and ruminating), and thus maintaining greater milk yield.

For the multiparous cows, there were much less evidence of any effect of CE supplementation on their response to regrouping. In fact, the results indicate some negative effects for the multiparous cows. Specifically, the multiparous CE cows had reduced lying duration for much of the period after regrouping compared to their baseline, whereas the multiparous control cows maintained their lying duration from their baseline. Few treatment differences for social behavior were observed for the multiparous cows. Interestingly, the multiparous CE focal exhibited reduced milk yield for 2 days following regrouping, whereas the multiparous control cow maintained their milk yield through that time period, potentially due to unknown effects.

Overall, the negative effects of regrouping (i.e. greater change of behavior and production compared with the baseline) were not as evident for the multiparous cows as they were for the primiparous cows. Researchers have demonstrated that primiparous cows may be more negatively affected by regrouping than multiparous cows (Mazer et al., 2020). That is probably due to regrouping increasing competition between cows for resources (i.e. feed, lying stalls, water) (von Keyserlingk et al. 2008; Schirmann et al., 2011) and primiparous cows having physical disadvantages (i.e. smaller body weight and size) to compete for those resources compared with multiparous cows (Moran and Doyle, 2015). Thus, a lesser effect of regrouping on the multiparous cows (as compared to the primiparous cows) may have been responsible for less of a detectable benefit of CE supplementation for the multiparous cows after regrouping.

3.2 Limitations and future research

One of the limitations of this research study was that it was not possible for each focal cow to be introduced to the exact same social structure, due to one of the non-focal cows needing to leave the treatment pens each week upon entry of the new focal cow. Further, each focal cow may have experienced a different social structure after regrouping, increasing variability in the dynamic situation between focal cows within each treatment. Ideally, each focal cow would have been added to her treatment pen, followed for 7 d, and then removed. In this way, the social dynamic of the 29 non-focal cows would have remained the same (more or less) for each treatment pen over the course of the study, with the only change being the new focal cow introduced each week. Although is difficult to measure the extent of the social structure effect had on the results of this study, the social structure effect was applied for both treatments pens, therefore, it is very unlikely that it had a major effect on the outcomes of this study.

Although this research measured several behavioral and production indicators (i.e. lying time, feeding time, milk production, etc.) to investigate the negative effects of regrouping on cows, it was still limited in what measures could be collected. To better quantify the effects of CE on the psycho-social stress of cows after regrouping, it would have been valuable to measure individual DMI, some physiological measure of stress (e.g. fecal cortisol), and milk components (to calculate energy corrected milk). In my study, it was not possible to measure individual DMI after regrouping as cows were fed at a common feed bunk in the treatment pens. Future studies should make use of individual feed bins to measure DMI. Individual feed bins would make it possible to follow the potential changes in DMI and feeding behavior over time after regrouping, potentially explaining the other changes observed (for example rumination time and milk production). Further, in those future studies it would also be of interest to measure the change of social and agonistic behavior between focal cows before and after regrouping, which was not possible in my study give that cows were individually housed during the baseline period.

In the current study we were only able to test one concentration of CE (4 g/d per cow). In other studies that have investigated supplementing several levels of CE to individuals, there were contradictory results. One of these studies reported that consuming a high concentration of CE caused feed aversion in piglets (Clouard et al., 2012), whereas, in another study it was demonstrated that consuming a high concentration of CE resulted in reduced anxiety in rats undergoing a stressful event (Faturi et al. 2010). Thus, a valuable addition to future research would be investigating supplementation of different concentrations of CE (i.e. high, medium, and low) on their ability to reduce anxiety in primiparous cows following regrouping.

Another potential limitation of my study is that I only enrolled 14 primiparous cows for both treatments. While I detected interesting findings for primiparous cows supplemented with CE, a greater sample size would have better corroborated my findings. Therefore, if researchers were to investigate the effects of supplementing different concentrations of CE to primiparous cows undergoing regrouping events they must include the indicators previously indicated in this section and have a greater sample size.

The use of CE for reducing the negative response to stress may have other applications in the dairy industry. Dairy cattle are constantly undergoing stressful situations in their routine (i.e. weaning, calving, hoof trimming, etc.). For example, weaning is probably one of the most stressful events that a dairy animal may experience, presenting a reduction in DMI and change of behavior for at least a few days post weaning. Future studies should be focused on investigating if supplementing CE would reduce the stress of weaning on calves or during other psycho-social stressful events.

3.3 Implications

Overall, the results of my thesis research suggest that regrouping may negatively affect behavior and milk production of dairy cows, providing further evidence that the effects of regrouping require mitigation. To our knowledge, this is the first study, to date, to investigate if the supplementation of CE to cows would reduce the negative effects of regrouping on cow behavior and production. Indeed, this thesis provides evidence that CE may have mitigated those negative effects of regrouping in primiparous dairy cows. Further, the results of this thesis work would suggest that farmers should limit excessive and unnecessary regrouping events of lactating cows to increase animal welfare. From these results, the supplementation of CE to primiparous cows would be recommended to dairy producers to reduce the negative effects of regrouping on behavior and milk production.

CHAPTER 4: REFERENCES

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