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Running Head: SEPARATION EFFECTS IN DAIRY CATTLE

The effect of conspecific removal on behavioral and physiological responses of dairy cattle

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ABSTRACT

Adverse social and welfare implications of mixing dairy cows or separating calves from their mothers have been documented previously. Here we investigated the behavioral and physiological responses of individuals remaining after conspecifics were removed. We conducted a series of four experiments incorporating a range of types of different dairy cattle groupings (Experiment 1[**E1**], 126 outdoor lactating dairy cows; Experiment 2 [**E2**], 120 housed lactating dairy cows; Experiment 3 [**E3**], 18 housed dairy calves, and Experiment 4 [**E4**], 22 housed dairy bulls) from which a subset of individuals were permanently removed (E1 n = 7, E2 n = 5, E3 n = 9, E4 n = 18). Associations between individuals were established using near-neighbor scores (based upon identities and distances between animals recorded prior to removal) in E1, E2 and E3. Behavioral recordings were taken for 3 to 5 d, before and after removal on a sample of cattle in all 4 experiments (E1 n = 20, E2 n = 20, E3 n = 9, E4 n = 4). In two experiments with relatively large groups of dairy cows, E1 and E2, the responses of cows that did and did not associate with the removed cows were compared. An increase in time that both non-associates and associates spent eating was observed after conspecific removal in E1. In E2 this increase was restricted to cows that had not associated with the removed cows. A reduction in ruminating in remaining cattle was observed in E3 and eating in E4. Immunoglobulin A concentrations increased after separation in both E3 and E4 cattle, but did not differ significantly between associates and non-associates in E2. Blood and milk cortisol concentrations were not affected by conspecific removal. These findings suggest that some animals had affected feeding behavior and IgA concentrations after removal of conspecifics.

Key Words: association, dairy cattle, separation, immunoglobulin A, conspecific

INTRODUCTION

Increasingly, animal emotions form the basis of animal welfare definitions (Dawkins, 1990; Fraser and Duncan, 1998; Mendl and Paul, 2004; Broom, 2010), with public concern for the welfare of farm animals often arising from the recognition that animals are able to experience emotions (Špinka, 2012, Boissy and Erhard, 2014). Farm animals are gregarious and their social environment plays a fundamental role in the individual's welfare status (Keeling and Gonyou, 2001; Rault, 2012), with many benefits being derived from the presence of a conspecific (Rault, 2012). Dairy cattle form long lasting social bonds (Reinhardt and Reinhardt, 1981; Færevik et al., 2006) and show strong affiliation to conspecifics (Holm et al., 2002). In modern production systems the regrouping of cattle (regrouping is defined here as a two-step process: 1. Separation from the old group and 2. Introduction to a new group) occurs frequently in order to create homogenous groups organized by common characteristics, such as age, milk yield, body condition, reproduction, and health status (Bøe and Færevik, 2003; Raussi et al., 2005). This regrouping process, in particular step 2, has been documented to result in social stress evidenced by behavioral changes that include increased aggression (Raussi et al., 2005), vocalizations (Boissy and Le Neindre, 1997; Færevik et al., 2006; De Paula Vieira et al., 2010), changes in locomotory behavior (Hasegawa et al., 1997; von Keyserlingk et al., 2008) and has negative impacts upon production traits, such as reduced feed intake (von Keyserlingk et al., 2008; De Paula Vieira et al., 2010; Schirmann et al., 2011; Duve et al., 2012), milk yield (Hasegawa et al., 1997; von Keyserlingk et al., 2008) and weight gain (De Paula Vieira et al., 2010). These negative effects have been documented across a range of cattle scenarios including lactating cows, heifers and bulls (Mench et al., 1990; Hasegawa et al., 1997; Mounier et al., 2006). However, studies investigating the effect of repeated regrouping show contradictory findings, with some suggesting cattle do habituate to regrouping over time (Mench et al., 1990) and others

72 providing no evidence of this (Raussi et al., 2005). Conceivably the stability of relationships
73 between the cattle and the number of animals affected by the regrouping may determine the
74 ability of cattle to habituate to the practice.
75
76 The majority of studies investigating the regrouping of cattle have focused on the effect on
77 the individual(s) being regrouped (e.g. Mench et al., 1990; Raussi et al., 2005; Mounier et al.,
78 2006; von Keyserlingk et al., 2008). In these studies the effects of separation are often hard
79 to distinguish from the effects of the novel environment (Rault, 2012). Although the impact
80 of new individuals introduced to a previously established group has been described, the
81 impact on the individual(s) remaining in the original group has not. In one study by
82 Schirmann et al. (2011) the difference in response to regrouping between cows that were
83 moved to a new pen and those that stayed in their home pen was investigated, however due to
84 the experimental design, the effects of removal of individual cows on those remaining in the
85 home pen could not be separated from the effects of the newly introduced cows.
86
87 Measurement of stress traditionally involves behavioral observation and physiological
88 evaluation of, for example, HPA activation (e.g. cortisol) or immunological response (e.g.
89 immunoglobulin A). Immunoglobulin A (IgA) represents a main element of the humoral
90 immune response, which provides protection against pathogens at mucosal surfaces (Snoeck
91 et al., 2006). In its secretory form (S-IgA) it serves to prevent infective agents such as
92 bacteria and viruses from breaching the mucosal barrier, whilst within serum it functions as
93 an inflammatory antibody acting on immune effector cells (Snoeck et al., 2006). Relatively
94 little information is available on the relationship between IgA and stress responses in farm
95 animals, with the exception that in pigs S-IgA reportedly increases as a result of chronic
96 stress caused by social isolation during the first 12 days and declines thereafter (Royo et al.,

2005). A similar response has been observed in dogs in the first six days following separation from a conspecific (Walker et al., 2014) and as a result of stress experienced upon entry into a kennel environment (Skandakumar et al., 1995). In response to acute stress, S-IgA levels in rats and dogs have been documented to decrease (Guhad and Hau, 1996; Kikkawa et al., 2003), and in humans a large body of evidence concludes that negative emotional valence, resulting from short-term stress, results in decreased S-IgA (reviewed by Segerstrom and Miller, 2004). Although the influence of emotional states on IgA secretion in cattle has not been examined, bovine IgA has been quantified in milk (Newby and Bourne, 1977; Honkanen-Buzalski and Sandholm, 1981), serum, lacteal, saliva, nasal and vaginal secretions (Duncan et al., 1972). Research has demonstrated that IgA in bovine milk is predominately serum derived (Newby and Bourne, 1977), suggesting that milk could act as an appropriate, non-invasive, accessible alternative to serum in the measurement of short and long term stress. Likewise, cortisol concentrations in milk from cows in established lactation have been demonstrated to directly relate to cortisol concentrations in blood (Shutt and Fell, 1985), suggesting that milk is a suitable substitute for serum when measuring cortisol concentrations in dairy cattle.

The objective of this study was to investigate the effect of step one of regrouping: The effect that the removal of individuals from the group has on remaining group members, utilizing behavior observations and two physiological measures; cortisol and immunoglobulin A (IgA).

MATERIALS AND METHODS

These experiments were approved by the University of Queensland Animal Ethic Committee, approval numbers CAWE139/10 and CAWE068/11.

122

123 ***Experiment 1.***

124 ***Animals.*** In Experiment 1 **(E1)**, Observations were made of a herd of 126 lactating
125 Holstein-Friesian and mixed breed dairy cows at the University of Queensland (Gatton,
126 Queensland, Australia). The study was carried out during mid-winter (mean temperature =
127 16°C ± 4.4°C) when the herd was maintained in a 1.93 ha outdoor feedlot area (Figure 1),
128 with a stocking density of 65.3 cow per ha. Of the total 126 cows, 55% (69/126) were
129 Holstein-Friesian; 27% (34/126) Holstein-Friesian crossbreed; and one (0.8%) each of Jersey;
130 Brown Swiss; Brown Swiss cross Jersey; Ayrshire cross and the remaining 15% (19/126)
131 were of unknown crossbreed.

132

133 The group structure was dynamic with cows temporarily removed from the herd as a result of
134 cessation of lactation, illness or estrus cycle, as well as for use during agriculture and
135 veterinary teaching demonstrations and practicals. The cows were milked twice daily in a
136 herringbone parlor between 0600 to 0800 h and 1500 to1800 h. Feed was delivered twice
137 daily at 0800 and 1300 h to a covered feeding trough in a paddock. The cows were
138 maintained on a TMR consisting of 13% soybean meal, 38% grain mix, 26% barley silage,
139 14% soybean silage, 6% lucerne silage and 3% mineral mix on a DM basis. The feed bunk
140 was 60 m long with enough room for all cows to feed comfortably at the same time. Water
141 was available *ad libitum.*

142

143 ***Near Neighbour Observations.*** A subset of 7 individuals were selected for removal
144 and subsequent culling due to age-related reductions in milk production. Nearest neighbor
145 identities and distances were recorded prior to this removal to establish the strength of
146 association between these removed individuals and others in the group. The distance from

each of the 7 individual cows signaled for removal, to up to 5 nearest neighbors was estimated visually using cow length (mid-point between the shoulder and tail of the cow shoulder to rump (Gibbons et al., 2010) as a guide. Near-neighbor observations were carried out on 34 to 39 (SD = 1.7) occasions, for each of the 7 focal animals, over 9 d in three differing locations: the paddock, order of entry into the milking parlor and during feeding. Recordings in the paddock were conducted between 0900 to 1300 h (with at least 1 h between recording sessions) on 21 to 24 (SD = 1.1) occasions/cow over 9 d. Order of entry into the milking parlor recordings were carried out on 3 to 5 (SD = 0.9) separate occasions per cow at the p.m. milking, with the cow on either side of each focal cow recorded. Cows at the end of a milking row had only one recorded neighbor. Feeding recordings followed the methodology described by Cooper et al. (2008), with cows each observed on 8 to10 (SD = 0.8) occasions across 9 d between 1300 to1500 h, following the delivery of the afternoon feed ration. The two nearest-neighbors on either side of each focal cow were recorded and considered feeding partners, providing they were within a distance of 1 cow length. If a cow was at the end of the feeding line she was considered to only have one feeding partner. Observations were carried out in these three locations as these were the only locations that the cows had access at set times across a 24 h day.

Selection of Experimental Subjects. To allocate subjects to an associate or a non-associate group we used the near-neighbor recordings to identify the 10 individuals that displayed the greatest (associate), and the 10 individuals that displayed the weakest (non-associate), association to the 7 individuals identified for removal. Probability theorem was used to establish that these interactions did not occur by chance (see statistical analysis section). The number (n = 10) of individuals allocated to both the associate and non-associate groups was determined from the natural social structure of cattle which is reported to be a

mean group size of 10-11 individuals (Bouissou et al., 2001). The 20 selected cows had mean age of 6.1 ± 1.8 (SD) yr and weight of 577 ± 144.5 (SD) kg. The mean milk yield per experimental cow was 27.1 ± 7.9 (SD) L per day during the course of the experiment. Stocking density after removal was 60 cows per ha.

Behavioral Observations. An ethogram (Table 1) was developed based on previous studies (Krohn, 1994; Fröberg and Lidfors, 2009; Fogsgaard et al., 2012). Behavioral observations were carried out by three trained observers, two of whom were recording at any one time. Observations were made continuously using focal animal sampling for each of the 20 cows, at 1h intervals, for a 10 min per cow duration. Cows were recorded in the same order each time. Observations were carried out continuously for 8 d between morning and afternoon milking from 0800 to 1500 h (70 min per day/per cow), 4 d being before removal of designated cows (d -5, -3, -2, -1) and the remaining 4 d after removal (d +1, +2, +3, +4). Removal took place at 1900 h, therefore the first observations after separation began after 13h post separation.

Experiment 2

Animals. Experiment 2 **(E2)** was carried out at the Estonian University of Life Sciences Maarja Farm, Tartu, Estonia. Observations were made of 2 herds each containing 60 lactating Holstein-Friesians, each with a stocking density of 0.12 cows per m^2. Cows in both herds were free-stall housed within the same building and fed cut grass in their housing (Figure 1). The total housing area for both herds was 1032 m^2. The group structure was dynamic with cows temporarily removed for cessation of lactation, illness, or estrus. Milking occurred twice daily in either a traditional parlor (n = 8) at 0530 and 1500 h or twice daily in a robotic milking system (n = 12). The two differing milking systems were engaged for

197 teaching demonstrations and research purposes. Cows had *ad libitum* access to water and
198 were fed a TMR consisting of grass/clover silage 50.8% DM, barley 24.6% DM, wheat 7.8%
199 DM, rapeseed cake 15% DM and mineral feed 1.8% DM. They were fed twice daily at 1000
200 and 1700 h. The parlor-milked cows had individual access to bins in their housing (Figure 1)
201 with varying amounts of added concentrate in TMR. Robot-milked cows received an
202 additional 0.88-2.64 kg DM/d of concentrate supplement (Baltic Argo AS, Tallinn, Estonia)
203 from an automated feeder and 2.64-5.28 kg DM/d in the robot, according to yield.
204
205 ***Near Neighbor Observations.*** A subset of 5 individuals (parlor milked herd n = 2;
206 robot milked herd n = 3), with a mean age of 3.8 ± 0.84 (SD) yr, were identified for removal
207 due to imminent dry-off and forthcoming parturition. Stocking density after removal in E2
208 was 0.11 cow per m^2 for both the parlor milked and robot milked herds. An additional subset
209 of 5 individuals (parlor milked herd n = 2; robot milked herd n = 3), with a mean age of 4.6 ±
210 1.1 (SD) yr, were randomly selected to aid establishment of a control group. Nearest neighbor
211 distances were determined for all 10 cows prior to removal of the subset of 5 individuals
212 mentioned above. The near neighbor observations were carried out in loose housing area
213 which included the feed bunk (Figure 1) utilizing the methodology described in E1, with the
214 exception that to be considered a near-neighbor the cow had to be within 1 cow length of the
215 focal animal (rather than three) due to the more intensive housing conditions. Observations
216 were not carried out separately during milking or at the feed bunk, as was done in E1, due to
217 the use of the robot milking system and the inclusion of the feed bunk in the loose housing
218 area. Near-neighbor observations were carried out on 12 occasions at 1300; 1900; 2400 and
219 0500 h across three consecutive days. These occasions represented even distribution across
220 each 24 h period whilst also incorporating a 2 h period after milking (parlor side) and feeding
221 before the start of an observation session.

222

223 ***Selection of Experimental Subjects.*** A subgroup of 20 individuals (parlor n = 8; robot
224 milkers n = 12) were selected as outlined below and divided into two groups of 10. Selection
225 utilized near-neighbor recordings to first identify the 10 individuals that displayed the
226 strongest association to the 5 individuals signaled for subsequent removal. These 10
227 individuals formed the associate group (mean age 3.3 ± 1.4 (SD) yr). To create a non-
228 associate group, 5 individuals from the herd (other than those signaled for removal) were
229 selected at random and the 10 individuals with the strongest near neighbor association to
230 these formed the non-associate group (mean age 4.0 ± 1.10 (SD) yr). This method of
231 selection differed from E1 to control for the possibility that our methodology in E1 may have
232 inadvertently selected cows that were less sociable to all cows, not just the removed cows.
233 Hence in E2 we chose cows that were equally sociable, but to different cows, for our non-
234 associate group.

235

236 ***Behavioral Observations.*** The same ethogram detailed in E1 was used in E2.
237 Behavioral observations of the selected subgroup of 20 individuals were carried out by the
238 lead researcher from a viewing platform above the housing area for 3 d (d -6, -4, -2) before
239 removal of pre-selected individuals and 3 d (d+1, +3, +6) after removal. Observations were
240 conducted using instantaneous scan sampling (Martin and Bateson, 1993) at 10 min intervals
241 for a 2 h duration at 0200, 1000 and 1800 h, totaling 36 scans per day.

242

243 ***Physiological Sample Collection.*** Milk samples were collected from each of the 20
244 cows, using polypropylene centrifuge tubes (BRAND GMBH + CO KG, Wertheim,
245 Germany), during p.m. milking between 1500 to 1600 h on d +1, +3, +6. Samples were

centrifuged immediately for 10 min at 3500 rpm, and skim milk was extracted and frozen at -25°C for subsequent determination of IgA and cortisol concentrations.

Experiment 3

Animals. Experiment 3 **(E3)** was carried out at the same farm as E2 with observations of 9 Holstein-Friesian dairy calves, maintained on the farm within a group of 18 calves (mean age 65 d ± 16.8 d [SD]). The group of 18 calves were housed together continuously in a straw and peat-bedded pen (4.5m x 3.5m; stocking density = 1.1 calf per m^2) (Figure 1) from approximately 2 wk after birth and maintained on milk replacer (Denka Milk, Voorthuizen, Netherlands) fed at levels up to 8 L/d at 8 wk of age, obtained from an automatic milk feeder. Levels of milk intake were not recorded. Hay was provided ad libitum and pellets (Saldus Labiba, Saldus, Lativa), were provided at up to 1.72 kg DM/d, according to live weight, accessed from an automatic feeder. All individuals had *ad libitum* access to water. A subset of 9, 8 wk old, calves were identified for removal into older age groupings. The remaining 9 calves were a mean age of 36 d ± 9.8 d (SD). The stocking density after removal was 0.6 calves per m^2. Near neighbor observations were not possible due to the small numbers of calves in this study.

Behavioral Observations. The same ethogram detailed in E1 was used in E3 with the addition of 'play' behavior. Behavioral observations of the 9 calves were carried out by a single observer from a viewing area fronting the housing area for 3 d (d -5, -3, -1) before and 3 d (d+1, +3, +6) after removal of pre-selected individuals. Observations were conducted using instantaneous scan sampling (Martin and Bateson, 1993) at 10 min intervals for 2 h at 1100 and 1700 h, totaling 24 scans per day

Physiological Sample Collection. As milk samples were unable to be collected from this group, blood samples were collected by a veterinarian from the coccygeal vein on each of the 9 calves, using heparinized tubes (Venoject ®, Terumo Corporation, Belgium), between 1000-1100 h on d -1, +1, +3, +6. Samples were centrifuged immediately for 20 min at 3500 rpm, and serum was extracted and frozen at -25°C for subsequent determination of IgA and cortisol concentrations.

Experiment 4

Animals. Experiment 4 **(E4)** was conducted on a commercial dairy farm in Rahinge, Estonia. Subjects comprised 4 16-month old, Holstein-Friesian bulls, maintained in a group of 22 (stocking density = 0.3 bulls per m^2). They had been housed together continuously since around 8 wk of age and were loose housed in a deep straw pen (70 m^2) within a larger barn (Figure 1). They had ad libitum access to water and were maintained on a TMR consisting of grass/clover silage, hay (83% DM) and commercial pellets (300 g/d, 86% DM), fed at 0900 and1700 h**.** A subset of 18 of the bulls were selected for removal based on qualification for live export. Stocking density after removal was 0.06 bulls per m^2.

Behavioral Observations. Observations were carried out from a viewing platform above the group utilizing the same ethogram as in E1. The 4 bulls were observed using focal animal sampling (Martin and Bateson, 1993) for 15 min durations, at 1 h intervals, between 1000 and 1800 h (totaling 2 h of per bull/ per day) on 4 d (d -7, -4, -2, -1) before and after (d +1, +3, +5, +7) removal**.** Near neighbor observations were not possible due to the small numbers of bulls in this study.

Physiological Sample Collection. As milk samples were unable to be collected from this group, blood samples were collected by a veterinarian from the coccygeal vein, using heparinized tubes (Venoject®, Terumo Corporation, Belgium) on each of the 4 bulls animals between 1000 and 1100 h on d -1, +1, +3, +5. The bulls were restrained in a holding corner of their home pen during sample collection. Samples were centrifuged immediately for 20 min at 3500 rpm, and serum was extracted and frozen at -25°C for subsequent determination of IgA and cortisol concentrations.

Identification

Individuals in all experiments were numerically identified (on both the head and rump) using tail paint (FIL Tell Tail, Farmers Industries Limited, New Zealand [E1] or Porcimark Maerkespray, Kruuse [E2, E3 and E4]).

Physiological Sample Analysis

To calculate IgA concentration, the optical density of samples was compared to the optical density of a standard with a known concentration of IgA, using the Bovine IgA ELISA Quantitation Kit (E103, Bethyl Laboratories, Montgomery, Texas, USA). ELISA plates were coated with 100 µl/well of diluted anti-bovine IgA antibody (E10-121, Bethyl Laboratories, Montgomery, Texas, USA) and incubated at room temperature for 60 min diluted to 1µg/mL in carbonate-bicarbonate buffer at pH 9.5. Plates were washed five times with wash solution (50m*M*/L Tris, 0.14*M*/L NaCl, 0.5ml/L Tween20, dH20). Plates were then blocked for 30 min at room temperature with 200 µl/well of blocking solution (50m*M*/L Tris, 0.14*M*/L NaCl, dH20, 1% BSA) added to each well and incubated at room temperature for 30 min. Plates were washed five times and 100µl/well of diluted standards or samples were added. Then 1.5µl of sample was diluted in 1.5mL of diluent, based on the expected concentration

(sample dilute: 50mM/L Tris, 0.14M/L NaCl, 0.5ml/L Tween20). Samples were diluted starting at 1:1000 and extending to 1:156,000. Plates were incubated at room temperature for a further 60 min and then washed five times, followed by the addition of 100 µl/well of diluted anti-bovine IgA horseradish peroxidase antibody E10-121, (Bethyl Laboratories, Montgomery, Texas, USA) and incubation at room temperature for a further 60 min. Plates were washed a further five times and 100 µl/well of tetramethylbenzidine substrate solution was added, with a stop solution (0.18M H_2SO_4 at 100µl/well) added after 5 min. Optical density was read at 450 nm with a microplate reader (IL650, Instrumentation Laboratory, Cheshire, UK). The concentration of IgA in each sample was calculated using linear regression from a standard curve generated from the standards using IL650 software. IgA results are reported in mg/dL of serum/milk.

Cortisol concentrations were quantified with a solid phase competitive chemiluminescent enzyme immunoassay (Immulite 1000, Siemens Medical Solutions Diagnostics, Camberley, UK) using a commercial kit (Immulite Cortisol Kit, Siemens Medical Solutions Diagnostics, Camberley, UK). Results for the cortisol were generated from the standard curves produced within the Immulite 1000. The cortisol detection limit was 27.6 m*M*/L.

All samples were analyzed by CTDS Veterinary Diagnostic Laboratory, Leeds, UK. The within assay coefficients of variability for serum and skim milk were 6.1 and 1.4% (cortisol) and 10.1 and 3.9% (IgA), respectively. The between assay coefficients of variability for serum and skim milk were 8.15 and 4.4% (cortisol) and 16.6 and 5.2% (IgA), respectively

341

342 ***Statistical Analysis***

343 All values are reported as means ± standard deviations. To establish the nearest neighbors in
344 E1, a clustering of the observations using a dendogram was performed. From this we
345 identified the 10 individual cows (associate group) that had the closest associations with the 7
346 focal cows signaled for removal. For the non-associate group the 10 cows with the weakest
347 associations with the focal cows were selected from the cluster analysis. To confirm that
348 these interactions did not simply occur by chance, the probability of an individual member of
349 the herd being recorded with one of the 7 focal cows on either 2 or 3 occasions out of the
350 total number of observations was calculated using probability theorem (Cooper et al., 2008).
351 To summarize, the chance of each focal cow interacting with any other cow on any
352 observation was 125/125 (125 cows were used as this was the number of cows any focal cow
353 could interact with). The chance of the focal cow interacting on the subsequent observation
354 with the same cow was 1/125, and with a different cow in the herd was 124/125. On the
355 second observation, the chance of the focal cow interacting with a different cow, except the
356 first or second, was 123/125 etc. This process continued until the remaining number of terms
357 was equal to the total number of observations that took place (e.g. 36 for focal cow 1). This is
358 numerically expressed as:

359

360 125/125 x 1/125 x 124/125 x 123/125 x 122/125….91/125 = 125!/90! (! = factorial)

361

362 The number of places cow 1 could appear was 36!/2!/34!
363 The probability of 2 cows occurring together on 2 occasions, out of a possible 36 is:
364 $125!/90! \times 1/125^{36} \times 36!/2!/34! = 0.026$

365

The probability of 2 cows being observed together on 3 occasions was similarly calculated as 125!/91! x $1/125^{36}$ x 36!/3!/33! = 0.003

In E2 and E3, Near Neighbor Associations were calculated using the association equation of Martin and Bateson (1993):

Near Neighbor Score =

$$\frac{\text{(number of times cow has been near-neighbor of focal cow)}}{\text{(number of focal cow observations)}}$$

Data was analyzed using Minitab (version 16). For all experiments, descriptive analysis of the recorded behavioral subcategories was carried out and any behavior occurring infrequently ($n < 3$) was removed. All behavior subcategories retained for analysis within each experiment (Table 1) were converted to a proportion per day due to variations in the total number of observations. The data mostly followed a normal pattern of distribution (Anderson Darling $P > 0.05$) and where deviations from normality occurred the data was transformed using Square Root or Log_{10} as appropriate. The different measures of behavior across the experiments precluded the data for being pooled and analyzed together. In E2 and E3 stand and walk behavior was combined. A General Linear Model (GLM) was used to investigate significant changes in behavior 'before' versus 'after' removal within each experiment. In E1and E2 the model included the variables before or after removal **(B/A),** day nested within B/A and group (associate or non-associate) nested within B/A. In E3 and E4 the model included B/A, each individual calf or bull and day nested within B/A. Post-hoc Tukey was engaged to investigate where significance lay across days in all models. A change in the concentrations of IgA and cortisol across days was investigated using linear regression.

RESULTS

Behavioral Responses

In E1, one cow was removed from analysis due to sickness in the final days of observation. Based on the number of times the focal (removed cow) was seen with one of the associate cows, the probability of association for each of the focal (removed) cows was follows: Focal cow (FC) 1 = 0.03, FC2 = 0.01, FC3 = 0.01, FC4 = 0.04, FC5 = 0.01, FC6 = 0.01, FC 7 =0.0002. None of the non-associate cows were recorded with the focal cows on more than one occasion. The chance that any 2 cows would be observed together on 2 or 3 occasions were small, 0.025 and 0.0035, respectively, therefore the nearest neighbor associations were considered real and not by chance. There was an increase (F = 9.47, df = 1, $P = 0.003$) in time spent eating (Table 2) after the separation, with no difference between the response of associate and non-associate cows ($P = 0.96$), which suggests that the removal of cows influenced remaining cows' eating behavior independent of measured associations between individuals (Table 2). There were no differences ($P \geq 0.24$) in time spent standing, walking, lying, sleeping, ruminating or butting after separation (Table 2). There was no effect of day ($P > 0.05$) on time spent performing any of the recorded behavior.

In E2, there was also an overall increase (F = 4.37, df = 1, $P = 0.04$) in time that remaining cows spent eating (Table 3) after separation, however, this was only in the non-associate cows (Tukey $P = 0.02$), not the associate cows (Tukey $P = 0.99$). There was no change ($P \geq 0.34$) in time spent in any other behavior after separation (Table 3). There was no effect of day ($P > 0.05$) on the occurrence of any behavior recorded.

In E3, a reduction in ruminating behavior was observed after separation (F = 7.97, df = 1, P = 0.007) (Table 4), with no change in time spent in any other behavior after separation. There was no effect of day (P > 0.05) on the occurrence of any behavior recorded in E3.

In E4, a decrease in eating was observed after separation in E4 (F = 4.94, df = 1, P = 0.037) (Table 5). Walking was increased on d+1 following separation, compared to d+3, +5, +7, -7 and -1 (F = 7.63, df = 6, $P < 0.0001$; all Tukey tests $P \leq 0.02$) (Figure 2). An effect of day was observed for standing (F = 2.69, df = 6, P = 0.04) and sleeping (F = 2.76, df = 6, P = 0.04), however post hoc Tukey tests were all non-significant ($P \geq 0.10$)

Physiological Responses

There was no difference in IgA concentration after separation between the non-associate and the associate group in E2 (mean non-associate group = 113.8 mg/dL; mean of associate group = 106.2 mg/dL, P = 0.42)

There was an increase in IgA over the first 6 days after separation in both E3 (mean before separation = 68.2 mg/dL; mean after separation = 83.7 mg/dL; SED = 0.78 mg/dL; F = 93.58, df = 1 P = 0.01) and E4 (mean before separation = 56.0 mg/dL; mean after separation = 88.3 mg/dL; SED = 3.96 mg/dL; F = 27.80, df = 1 P = 0.03) (Figure 3a and 3b). The regression equations, with adjusted r^2 and P values for the coefficients for day, were:

E3: $y = 62.0 (\pm 2.02) + 7.13 (\pm 0.74) x$, r^2 (adjusted) = 96.9%, P = 0.01

E4: $y = 43.0 (\pm 6.30) + 13.2 (\pm 2.51) x$, r^2 (adjusted) = 89.9%, P = 0.03

where y = IgA concentration in serum (mg/dL) and x = day

435

436 Cortisol concentrations did not differ in E2 between non-associate (mean = 1.8 m*M*/L) and
437 associate groups (mean = 1.7 m*M*/L; SED = 0.25 m*M*/L; *P* = 0.79), E3 (mean before
438 separation = 33.9 m*M*/L; mean after separation = 30.1 m*M*/L; SED = 1.33 m*M*/L; *P* = 0.19)
439 or E4 (mean before separation = 27.6 m*M*/L; mean after separation = 32.3 m*M*/L; SED = 2.87
440 m*M*/L; *P* = 0.30).

441

442 **DISCUSSION**

443 Social behavior is a major determinant of farm animal welfare (Keeling and Gonyou, 2001;
444 Rault, 2012). In cattle, social behavior is characterised by the formation and maintenance of
445 cohesive social groups (Gibbons et al., 2010). In the present study, across 4 experiments, we
446 removed both small groups of individuals from larger groups (less than 10% of the total
447 group) and large groups of individuals (50%-80% of the total group) to study the effect of
448 removal on remaining group members. Overt behavioral responses were limited and
449 physiological responses were restricted to increases in IgA concentrations in small groups of
450 cattle, with no evidence of cortisol responses. This indicates that, unlike moving individual
451 cattle into a new-group, acute stress was not experienced by the remaining cattle, even when
452 the majority of animals were removed and supports previous studies suggesting that cattle,
453 moving to new groups, habituate to repeated regroupings (e.g. Mench et al., 1990)

454

455 The observed increase in time spent eating after removal in our first two experiments (E1 and
456 E2) may be the result of some dominant cows being removed from the herd, allowing
457 subordinates more opportunities and time to feed than previously. Heifers, for example, have
458 been demonstrated to spend more time eating and ingest greater quantities of feed when they
459 are kept separate from older cows (Bøe and Færevik, 2003). Age is an important determinant

460 in social positioning (Kabuga, 1992) and different types of separation, e.g. dividing a feed
461 trough with protective barriers, have been used to improve the feeding time of subordinate
462 cows (Bouissou et al., 2001). In E2 removed cows were on average six months older than the
463 remaining associate cows. It is also possible that this increase could have resulted from more
464 space being available at the feed trough or that the cows were more active, resulting in
465 increased energy expenditure and subsequently intake requirements. However, this was not
466 supported by increased time spent walking.
467
468 In E1, the increase was observed in both the non-associate and associate groups and suggests
469 a herd effect. It was evident that the cows in the non-associate group did not associate with
470 the removed cows nor did they associate much with all other cows. Hence the decision was
471 made to change the non-associate group in E2 to be cows that associated with the same
472 frequency with cows that were not removed. In this instance the increase in feed intake was
473 confined to these non-associate cows, which may suggest feeding behavior in the associate
474 cows was suppressed, comparative to the non-associate cows, as a result of the removal of
475 associated cows. In addition, social buffering and/or emotion contagion could explain in part
476 the apparent absence of observed behavioral changes. Central to sustaining good welfare for
477 herd-living animals is the maintenance of synchronicity of behavior (Miller and Wood-Gush,
478 1991). From an evolutionary perspective, similarity in emotional states achieved via the
479 sharing of emotions can be seen as advantageous, as it results in efficient coordination of
480 behavior (Špinka, 2012). Social buffering refers to observed reduced arousal, during stressful
481 events, as a result of social grouping (Bouissou et al., 2001). Emotion contagion causes
482 animals to shift their own affective state to that of other animals in a particular state (Špinka,
483 2012). Although understudied, animals have been evidenced to both emit and detect
484 emotional signals, and during stressful events the social group can lower the individual's

485 arousal (Bouissou et al., 2001). The emotional sharing and synchronicity of behavior has
486 been most extensively studied in the social transmission of fear, which can be prevented by
487 the presence of companions that do not show fear or vice versa (Veissier and le Neindre,
488 1992; Mounier et al., 2006). For example heifers show less avoidance of unusual noise in the
489 presence of pen mates (Boissy and Le Neindre, 1990), appear less fearful in novel
490 environments when social partners are present (Veissier and le Neindre, 1992), display
491 increased fear in either feeding or explorative situations when exposed to urine from stressed
492 conspecifics and show a lower tendency to feed in the presence of a stressed partner, than in
493 the presence of an unstressed one (Boissy et al., 1998). In the present study it is possible that
494 individuals in the associate groups E1 and E2 did not show overt behavioral signs of social
495 stress because the majority of the group did not experience social separation or display
496 behavioral changes.
497
498 The natural social structure of cattle is that of a group with a mean size of 10-11 individuals
499 (Bouissou et al., 2001). Group living involves the formation of social relationships and
500 preferential interactions with certain companions (Nicol, 2011), which suggests cattle are
501 likely to form positive social relationships with more than one other individual. Social
502 support often provides a single partner to an individual (Færevik et al., 2006; De Paula Vieira
503 et al., 2010; Duve et al., 2012), however, as cattle naturally maintain larger groups, they
504 require more peers to benefit from social support (Boissy et al., 1998). It is possible that the
505 impact of the removal of a group of animals was diminished by the presence of other socially
506 important individuals that remained in the herd.
507
508 In E3 and E4 we observed behavioral changes that are more commonly associated with social
509 stress. In E3 and E4 ruminating and eating times were reduced, respectively. Unlike the first

two experiments, in E3 and E4 the groups were less fluid and the majority of group members were removed so that the remaining group members (e.g. E3 n=9, E4 n=4) experienced a more similar situation to previous isolation studies where one or a small number of individuals are isolated from a larger group (von Keyserlingk et al., 2008). Consequently, remaining peers may not have been present in large enough numbers to effectively buffer the negative effect of separation, and previous experiences of separation may not have occurred with enough frequency to facilitate habituation. Following regrouping, calves housed in large groups (group size n=16) have been shown to modify their behavior to indicate improved welfare, compared to calves housed in small groups (group size n=4) (Færevik et al., 2007). Furthermore, in E3 feed intake was restricted by automated feeding equipment which may explain why a reduction in rumination was observed and not feed intake.

The possibility that the behavioral observations in E3 and E4 were indicative of stress is supported by the approximately linear increase in IgA observed up to 6 days after separation. Although more research is required to confirm the comparative IgA concentrations in bovine milk versus serum, published research in other mammal species including, sows, dogs and rats, has demonstrated that milk is rich in IgA (e.g. Heddle and Rowley, 1975; McGhee et al., 1975; Klobasa et al., 1987). One study by Näslund et al., (2000) demonstrated a high correlation between IgA titres in milk and serum, supporting the possibility of milk acting as a non-invasive alternative to serum. Additionally, IgA levels in milk appear to be consistent across lactational stages (Näslund et al., 2000). This suggests that it was the experimental design (group size and separation), rather than the substrate within which IgA was quantified, that was responsible for the observed (or lack of) changes in IgA after removal. Conversely, as we did not include a control in this study we cannot conclusively rule out the possibility that other variables (e.g. age, health status or environmental changes) may account for the

increase in IgA observed. To the authors knowledge all subjects were in healthy condition, had not recently received vaccination, nor were there extreme weather changes during the course of the experiment or any other notable stimuli that may have influenced IgA levels.

We also measured cortisol both between groups and before and after removal and found no significant changes, which may be because social separation is a chronic stressor. Cortisol has previously been used as a measure of the physiological impact (through HPA arousal) of social separation in a range of species and across a range of social relationships (Hennessy, 1997). These have predominately involved brief separations and have not always resulted in activation of the HPA system (Hennessy, 1997). As serum collection was obtained more than 12 h after removal in the present study, it is possible that HPA arousal had occurred but ended.

CONCLUSIONS

Behavioral responses to removal of predetermined individuals were observed in four experiments to be context specific. In two experiments with relatively large groups of dairy cows, social stress post-separation appears to have been diminished by social buffering or previous habituation to separation. In two experiments with smaller groups of cattle, IgA concentrations and nutritional responses suggest that social separation resulted in stress over several days. These responses suggest that social separation can be detrimental to the welfare of those remaining in the group but that the impact can be ameliorated by the presence of unstressed group members.

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REFERENCES

Bøe, K. E., and G. Færevik. 2003. Grouping and social preferences in calves, heifers and cows. Appl. Anim. Behav. Sci. 80:175-190. http://dx.doi.org/10.1016/S0168-1591(02)00217-4

Boissy, A., and H. W. Erhard. 2014. How studying interactions between animal emotions, cognition, and personality can contribute to improve farm animal welfare. Pages 81-113 in Genetics and the Behavior of Domestic Animals. 2nd ed. T. Grandin and M. J. Deesing, ed. Academic Press, San Diego.

Boissy, A., and P. Le Neindre. 1990. Social influences on the reactivity of heifers: Implications for learning abilities in operant conditioning. Appl. Anim. Behav. Sci. 25:149-165. http://dx.doi.org/10.1016/0168-1591(90)90077-Q

Boissy, A., and P. Le Neindre. 1997. Behavioral, cardiac and cortisol responses to brief peer separation and reunion in cattle. Physiol. Behav. 61:693-699. http://dx.doi.org/10.1016/S0031-9384(96)00521-5

Boissy, A., C. Terlouw, and P. Le Neindre. 1998. Presence of cues from stressed conspecifics increases reactivity to aversive events in cattle: Evidence for the existence of alarm substances in urine. Physiol. Behav. 63:489-495. http://dx.doi.org/10.1016/S0031-9384(97)00466-6

Bouissou, M. F., A. Boissy, P. Le Neindre, and I. Veissier. 2001. The social behaviour of cattle. in Social Behaviour in Farm Animals. L. J. Keeling and H. Gonyou, ed. CABI, Wallingford, UK.

Broom, D. M. 2010. Cognitive ability and awareness in domestic animals and decisions about obligations to animals. Appl. Anim. Behav. Sci. 126:1-11. http://dx.doi.org/10.1016/j.applanim.2010.05.001

Cooper, M. D., D. R. Arney, C. R. Webb, and C. J. C. Phillips. 2008. Interactions between housed dairy cows during feeding, lying, and standing. J Vet Behav 3:218-227. http://dx.doi.org/10.1016/j.jveb.2007.09.005

Dawkins, M. S. 1990. From an animal's point of view: Motivation, fitness, and animal welfare. Behav Brain Sci 13:1-9. http://dx.doi.org/10.1017/S0140525X00077104

De Paula Vieira, A., M. A. G. von Keyserlingk, and D. M. Weary. 2010. Effects of pair versus single housing on performance and behavior of dairy calves before and after weaning from milk. J. Dairy Sci. 93:3079-3085. http://dx.doi.org/10.3168/jds.2009-2516

Duncan, J. R., B. N. Wilkie, F. Hiestand, and A. J. Winter. 1972. The serum and secretory immunoglobulins of cattle: Characterization and quantitation. J. Immunol. 108: 965-976.

Duve, L. R., D. M. Weary, U. Halekoh, and M. B. Jensen. 2012. The effects of social contact and milk allowance on responses to handling, play, and social behavior in young dairy calves. J. Dairy Sci. 95:6571-6581. http://dx.doi.org/10.3168/jds.2011-5170

Færevik, G., I. L. Andersen, M. B. Jensen, and K. E. Bøe. 2007. Increased group size reduces

conflicts and strengthens the preference for familiar group mates after regrouping of weaned dairy calves (*Bos taurus*). Appl. Anim. Behav. Sci. 108:215-228. http://dx.doi.org/10.1016/j.applanim.2007.01.010

Færevik, G., M. B. Jensen, and K. E. Bøe. 2006. Dairy calves social preferences and the significance of a companion animal during separation from the group. Appl. Anim. Behav. Sci. 99:205-221. http://dx.doi.org/10.1016/j.applanim.2005.10.012

Fogsgaard, K. K., C. M. Røntved, P. Sørensen, and M. S. Herskin. 2012. Sickness behavior in dairy cows during Escherichia coli mastitis. J. Dairy Sci. 95:630-638. http://dx.doi.org/10.3168/jds.2011-4350

Fraser, D., and I. J. H. Duncan. 1998. 'Pleasures','pains' and animal welfare: Toward a natural history of affect. Anim. Welf. 7:383-396.

Fröberg, S., and L. Lidfors. 2009. Behaviour of dairy calves suckling the dam in a barn with automatic milking or being fed milk substitute from an automatic feeder in a group pen. Appl. Anim. Behav. Sci. 117:150-158. http://dx.doi.org/10.1016/j.applanim.2008.12.015

Gibbons, J. M., A. B. Lawrence, and M. J. Haskell. 2010. Measuring sociability in dairy cows. Appl. Anim. Behav. Sci. 122:84-91. http://dx.doi.org/10.1016/j.applanim.2009.11.011

Guhad, F. A., and J. Hau. 1996. Salivary IgA as a marker of social stress in rats. Neurosci. Lett. 216:137-140. http://dx.doi.org/10.1016/0304-3940(96)13037-8

Hasegawa, N., A. Nishiwaki, K. Sugawara, and I. Ito. 1997. The effects of social exchange between two groups of lactating primiparous heifers on milk production, dominance order, behavior and adrenocortical response. Appl. Anim. Behav. Sci. 51:15-27. http://dx.doi.org/10.1016/S0168-1591(96)01082-9

Heddle, R. J., and D. Rowley. 1975. Dog immunoglobulins. I. immunochemical characterization of dog serum, parotid saliva, colostrum, milk and small bowel fluid. Immunology. 29: 185-195.

Hennessy, M. B. 1997. Hypothalamic-Pituitary-Adrenal Responses to Brief Social Separation. Neurosci Biobehav Rev. 21:11-29. http://dx.doi.org/10.1016/S0149-7634(96)00013-9

Holm, L., M. B. Jensen, and L. L. Jeppesen. 2002. Calves' motivation for access to two different types of social contact measured by operant conditioning. Appl. Anim. Behav. Sci. 79:175-194. http://dx.doi.org/10.1016/S0168-1591(02)00137-5

Honkanen-Buzalski, T., and M. Sandholm. 1981. Association of bovine secretory immunoglobulins with milk fat globule membranes. Comp. Immuno.l Microbiol. Infect. Dis. 4: 329-342. doi: http://dx.doi.org/10.1016/0147-9571(81)90019-9

Kabuga, J. D. 1992. Social relationships in N'dama cattle during supplementary feeding. Appl. Anim. Behav. Sci. 34:285-290. http://dx.doi.org/10.1016/S0168-1591(05)80089-9

Keeling, L. J., and H. Gonyou. 2001. Social Behaviour in Farm Animals. CAB International, Wallingford, UK.

Kikkawa, A., Y. Uchida, T. Nakade, and K. Taguchi. 2003. Salivary secretory IgA concentrations in Beagle dogs. J. Vet. Med. Sci. 65:689-693.

Klobasa, F., E. Werhahn, and J. E. Butler. 1987. Composition of sow milk during lactation. J. Anim. Sci, 64: 1458-1466. doi: 10.2134/jas1987.6451458x

Krohn, C. C. 1994. Behaviour of dairy cows kept in extensive (loose housing/pasture) or intensive (tie stall) environments. III. Grooming, exploration and abnormal behaviour. Appl. Anim. Behav. Sci. 42:73-86. http://dx.doi.org/10.1016/0168-1591(94)90148-1

Martin, P., and P. Bateson. 1993. Measurig Behaviour: An Introductory Guide, 2nd ed. Cambridge University Press, UK.

McGhee, J. R., S. M. Michalek, and V. K. Ghanta. 1975. Rat immunoglobulins in serum and secretions: Purification of rat IgM, IgA, and IgG and their quantitation in serum, colostrum, milk and saliva. Immunochemistry. 12: 817-823. doi: http://dx.doi.org/10.1016/0019-2791(75)90146-9

Mench, J. A., J. C. Swanson, and W. R. Stricklin. 1990. Social stress and dominance among group members after mixing beef cows. Can. J. Anim. Sci. 70:345-354. http://dx.doi.org/10.4141/cjas90-046

Mendl, M., and E. S. Paul. 2004. Consciousness, emotion and animal welfare: insights from cognitive science. Anim. Welf. 13:17-25.

Miller, K., and D. G. M. Wood-Gush. 1991. Some effects of housing on the social behaviour of dairy cows. Anim. Sci. 53:271-278. http://dx.doi.org/10.1017/S0003356100020262

Mounier, L., I. Veissier, S. Andanson, E. Delval, and A. Boissy. 2006. Mixing at the beginning of fattening moderates social buffering in beef bulls. Appl. Anim. Behav. Sci. 96:185-200. http://dx.doi.org/10.1016/j.applanim.2005.06.015

Muneta, Y., T. Yoshikawa, Y. Minagawa, T. Shibahara, R. Maeda, and Y. Omata. 2010. Salivary IgA as a useful non-invasive marker for restraint stress in pigs. J. Vet. Med. Sci. 72:1295-1300.

Näslund, K., M. Tråvén, B. Larsson, A. Silván, and N. Linde. 2000. Capture ELISA systems for the detection of bovine coronavirus-specific IgA and IgM antibodies in milk and serum. Vet. Microbiol. 72: 183-206. doi: http://dx.doi.org/10.1016/S0378-1135(99)00208-4

Newby, T. J., and J. Bourne. 1977. The nature of the local immune system of the bovine mammary gland. J. Immunol. 118: 461-465.

Nicol, C. 2011. Behaviour as an indicator of animal welfare. Pages 31-68 in Management and Welfare of Farm Animals. J. Webster, ed. Wiley-Blackwell, Chichester, UK.

Rault, J. L. 2012. Friends with benefits: Social support and its relevance for farm animal welfare. Appl. Anim. Behav. Sci. 136:1-14. http://dx.doi.org/10.1016/j.applanim.2011.10.002

Raussi, S., A. Boissy, E. Delval, P. Pradel, J. Kaihilahti, and I. Veissier. 2005. Does repeated regrouping alter the social behaviour of heifers? Appl. Anim. Behav. Sci. 93:1-12. http://dx.doi.org/10.1016/j.applanim.2004.12.001

Royo, F., K. Lyberg, K. S. P. Abelson, H. E. Carlsson, and J. Hau. 2005. Effect of repeated confined single housing of young pigs on faecal excretion of cortisol and IgA. Scand J Lab Anim Sci 32:33-37.

Schirmann, K., N. Chapinal, D. M. Weary, W. Heuwieser, and M. A. G. von Keyserlingk. 2011. Short-term effects of regrouping on behavior of prepartum dairy cows. J. Dairy Sci. 94:2312-2319.http://dx.doi.org/10.3168/jds.2010-3639

Segerstrom, S. C., and G. E. Miller. 2004. Psychological stress and the human immune system: A meta-analytic study of 30 years of inquiry. Psychol. Bull. 130:601-630.

Shutt, D. A., and L. R. Fell. 1985. Comparison of total and free cortisol in bovine serum and milk or colostrum. J. Dairy. Sci. 68: 1832-1834. doi: http://dx.doi.org/10.3168/jds.S0022-0302(85)81035-3

Skandakumar, S., G. Stodulski, and J. Hau. 1995. Salivary IgA: A possible stress marker in dogs. Anim. Welf. 4:339-350.

Snoeck, V., I. R. Peters, and E. Cox. 2006. The IgA system: a comparison of structure and function in different species. Vet. Res. 37:455-467. http://dx.doi.org/10.1051/vetres:2006010

Špinka, M. 2012. Social dimension of emotions and its implication for animal welfare. Appl. Anim. Behav. Sci. 138:170-181. http://dx.doi.org/10.1016/j.applanim.2012.02.005

Veissier, I., and P. le Neindre. 1992. Reactivity of Aubrac heifers exposed to a novel

environment alone or in groups of four. Appl. Anim. Behav. Sci. 33:11-15. http://dx.doi.org/10.1016/S0168-1591(05)80079-6

von Keyserlingk, M. A. G., D. Olenick, and D. M. Weary. 2008. Acute behavioral effects of regrouping dairy cows. J. Dairy Sci. 91(3):1011-1016. http://dx.doi.org/10.3168/jds.2007-0532

Walker, J., N. Waran, and C. J. C. Phillips. 2014. The effect of conspecific removal on the behaviour and physiology of pair-housed shelter dogs. Appl. Anim. Behav. Sci. 158: 46-56. http://dx.doi.org/10.1016/j.applanim.2014.06.010

Tables

Table 1: Behavioral categories and subcategories and their definitions

Behavior	Definition
Locomotive Behavior	
Walk[1,2,3,4]	Forward movement with three legs remaining in contact with the ground.
Stand[1,2,3,4]	All four hooves on ground and legs upright and extended to support body.
Lie[1,2,3,4]	Recumbent, with body on floor.
Sleep[1,2,3,4]	Sternally recumbent with head tucked backwards towards shoulder, resting on body.
Maintanence Behavior	
Eat[1,3,4]	Cow ingests (or pokes with muzzle) food provided at feed bunk or from automatic feeder. Calf ingests milk provided by automated milker.
Drink[4]	Cow imbibes from automated water system.
Defecate[5]	Cow passes a faecal motion in standing or lying position.
Urinate[5]	Cow passes urine in a standing or lying position.
Ruminate[1,2,3,4]	Regurgitation, chewing, and swallowing of food bolus.
Vocal Behavior	
Vocalise[5]	Audible noise is produced by the cow.
Oral Behavior	
Lick[5]	Part of the tongue is protruded and moved over surfaces (e.g. pen bars).
Sniff[5]	Muzzle close to an object (or other individual) and inhaling air.
Social Interaction	
Play[3]	Running, trotting, galloping or jumping, alone or with other calves.
Human Interaction[5]	Physical contact with human.
Butting[1]	Cow uses head to head or head to body contact in an attempt to physically push another cow, or cause a cow to rise from lying position.
Other	
Groom – Auto[2,3,4]	Rubbing parts of the body or head against other body parts or fixtures of the stall (including automated groomer), or licking body parts.
Groom – Allo[2,3,4]	Rubbing parts of the body or head against another individually or licking the other individual.

Table 1 provides behavioral descriptors utilized for analysis
[1] = Behaviors analyzed in Experiment 1
[2] = Behaviors analyzed in Experiment 2
[3] = Behaviors analyzed in Experiment 3
[4] = Behaviors analyzed in Experiment 4
[5] = Behaviors not included in analysis due to infrequency of occurrence

Table 2: The mean % (and SED) of time for each behavior occurring across all days before and all days after removal for non-associate cows (n = 10) and associate cows (n = 10) in Experiment 1 (investigation of the effects on remaining cows as a result of removal of a small group of individuals [n = 7] from the larger herd [n = 126]) and the probabilities of differences before and after removal. There were no significant (P ≤ 0.05) associate Vs non-associate effects or individual day effects

	Before Removal		After Removal			
Behavior	non-associate	associate	non-associate	associate	SED	Before-After P-Values
Standing	46.0%	55.5%	49.8%	56.3%	2.02	0.40
Walking	2.2%	2.4%	2.4%	2.9%	1.01	0.41
Lying	49.1%	41.1%	44.4%	39.3%	2.01	0.24
Sleeping	2.2%	1.0%	1.0%	1.5%	0.40	0.55
Eating	19.9%	19.8%	26.0%	26.7%	1.5	0.003
Ruminating	30.9%	31.6%	36.9%	30.5%	1.48	0.24
Butting	0.08%	0.10%	0.09%	0.11%	0.01	0.74

Table 3: The mean % (and SED) of recordings for each behavior occurring both before and after cow removal for non-associate cows (n = 10) and associate cows (n = 10) in Experiment 2 and the probabilities of differences before and after removal. Post hoc (Tukey P-Value) associates Vs non-associate effects are reported. There were no significant (P ≤ 0.05) individual day effects.

	Before Removal			After Removal				
Behavior	non-associate	associate	Tukey P-Value	non-associate	associate	Tukey P-Value	SED	Before-After P-Values
Standing	17.2%	17.2%	-	16.4%	15.7%	-	4.52	0.48
Lying	50.2%	50.9%	-	46.6%	49.4%	-	1.88	0.34
Sleeping	5.6%	4.4%	-	5.7%	3.5%	-	0.45	0.89
Eating	13.5%	16.2%	0.02	19.5%	16.7%	0.99	1.18	0.04
Ruminating	30.2%	32.4%	-	30.7%	32.1%	-	1.41	0.99
Grooming	1.16%	1.16%	-	1.35%	0.83%	-	0.24	0.82

Table 4: The mean % (and SED) of recordings for each behavior occurring in calves (n = 49) both before and after removal of conspecifics (n = 18) in Experiment 3. There were no significant (P ≤ 0.05) individual day effects.

Behavior	Before Removal	After Removal	SED	Before-After P-Values
Standing	20.1%	18.7%	2.37	0.50
Lying	51.7%	56.9%	2.96	0.17
Sleeping	28.0%	24.0%	2.64	0.40
Eating	9.4%	8.4%	3.17	0.99
Ruminating	28.85	20.1%	2.24	0.007
Grooming	2.12%	3.23%	2.45	0.29

Table 5: The mean % (±SED) of time for each behavior occurring in bulls (n = 4) both before and after removal in Experiment 4 and the probabilities of differences before and after removal and day.

Behavior	Before Removal	After Removal	SED	Before-After P-Values	Day P-Values
Standing	33.1%	32.8%	3.60	0.95	0.04
Walking	1.5%	1.2%	0.22	0.39	<0.0001
Lying	59.0%	55.1%	3.84	0.48	0.29
Sleeping	4.4%	6.6%	1.21	0.21	0.04
Eating	15.7%	8.8%	2.22	0.04	0.15
Drinking	1.3%	1.3%	0.48	0.98	0.46
Ruminating	36.3%	25.5%	4.59	0.11	0.41
Butting	0.9%	2.9%	1.06	0.19	0.30
Grooming	1.7%	3.1%	0.52	0.65	0.07

Walker Figure 1

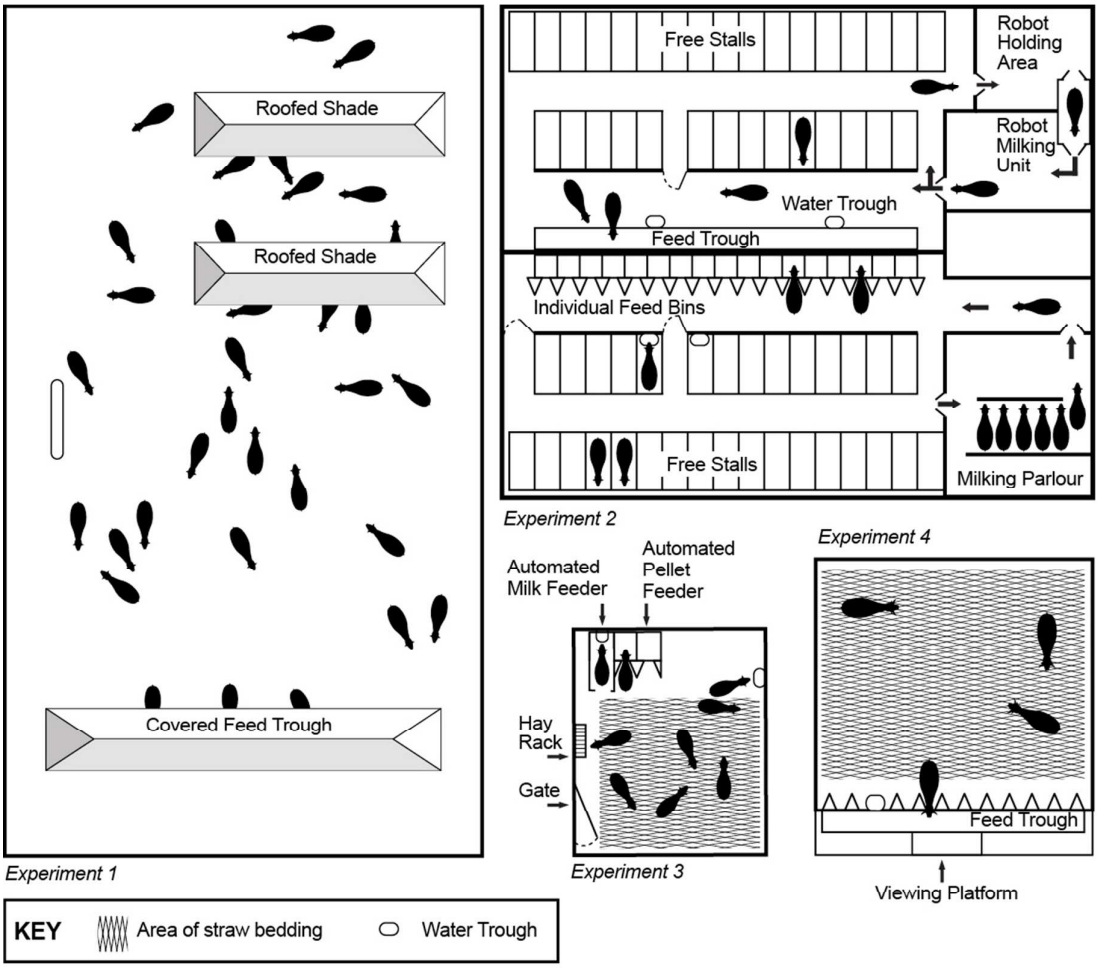

Figure 1, Manuscript Number , 33

Walker Figure 2

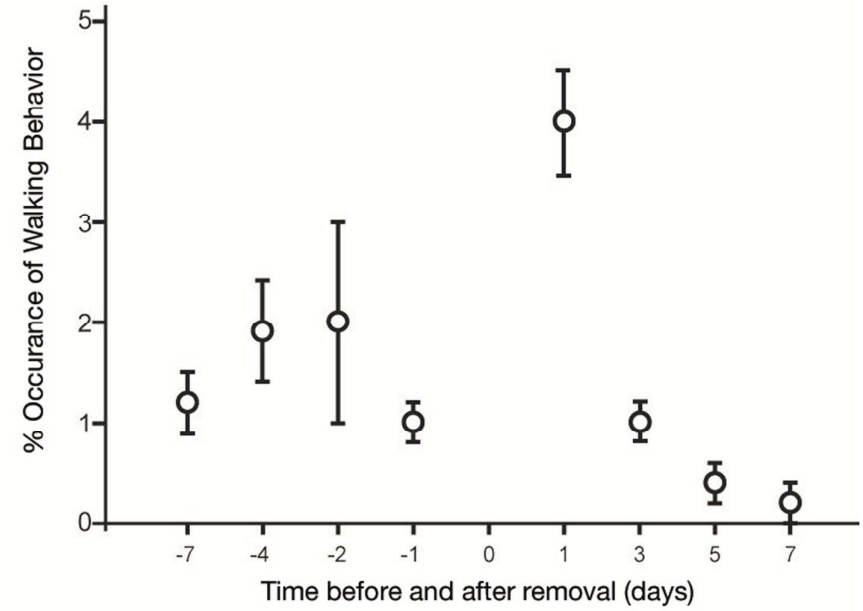

Figure 2, Manuscript Number , 34

Walker Figure 3

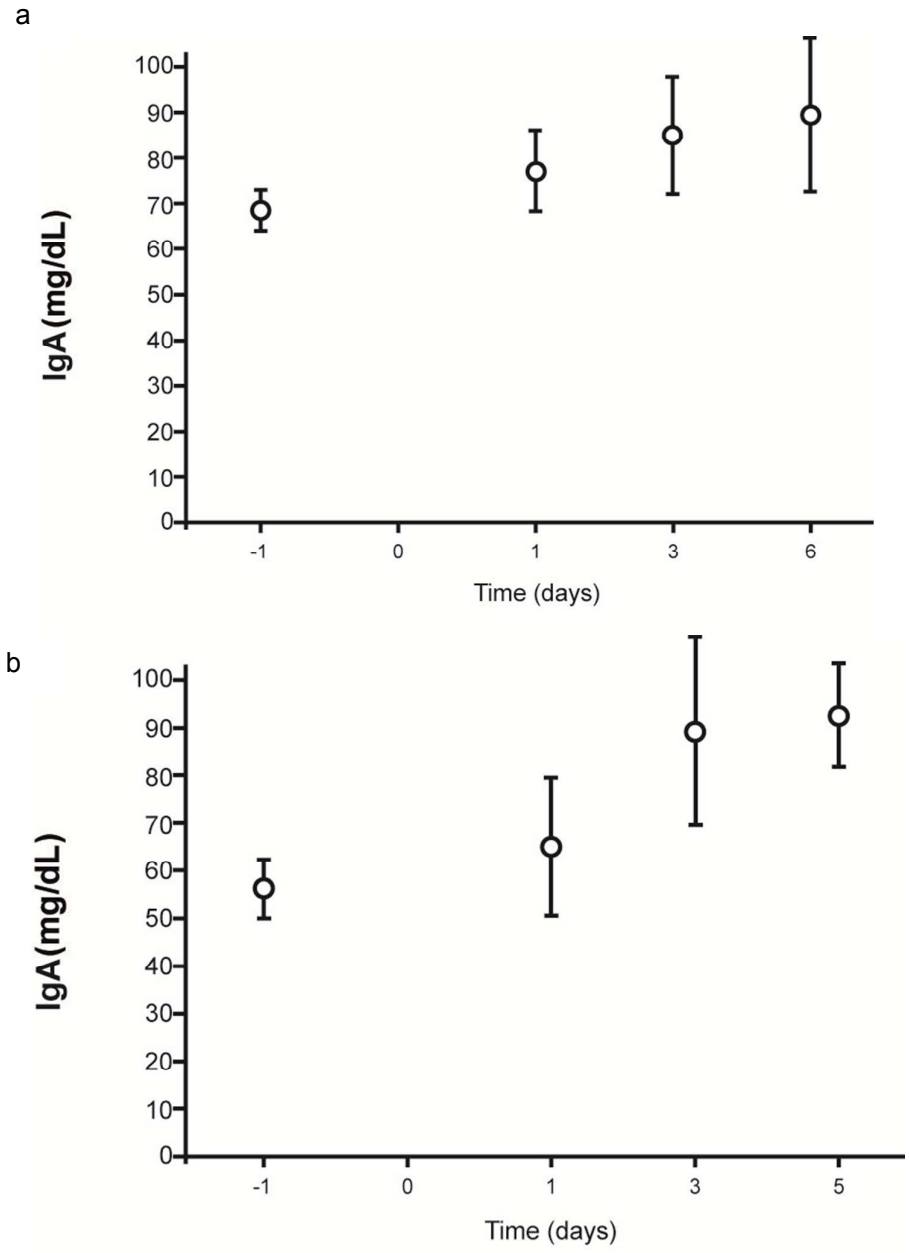

Figure 3, Manuscript Number , 35

Figure Captions

Figure 1: Overhead view of experimental facilities. Number of cattle does not represent exact experimental numbers in Experiment 1 and 2.

Figure 2: The % of walking behavior displayed across the 8 days of observations in Experiment 4.

Figure 3: Mean IgA concentrations in Experiment 3 a ($F = 93.58$, $df = 1$ $P = 0.01$) from d -1 (before removal) to d +1, +3, and +6 (after removal) and in Experiment 4 b ($F = 27.80$, $df = 1$ $P = 0.03$) across d -1 (before removal) to d+1, +3, and +5 (after removal).