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Social behavior and kin discrimination in a mixed group of cloned and non cloned heifers (*Bos taurus*)

M. Coulon[a,c,*], C. Baudoin[a], H. Abdi[b], Y. Heyman[c], B.L. Deputte[a,d]

[a] *Université Paris 13, Laboratoire d'Ethologie Expérimentale et Comparée, 99 avenue J-B. Clément, F-93430 Villetaneuse, France*
[b] *School of Behavioral and Brain Sciences, The University of Texas at Dallas, MS: GR4.1, 800 West Campbell Road, Richardson, TX 75080-3021, USA*
[c] *INRA, Biologie du Développement et Reproduction, F-78352 Jouy-en-Josas, France*
[d] *Ecole Nationale Vétérinaire d'Alfort, Département d'Ethologie, F-94704 Maisons-Alfort, France*

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Abstract

For more than ten years, reproductive biotechnologies using somatic cell nuclear transfer have made possible the production of cloned animals in various domestic and laboratory species. The influence of the cloning process on offspring characteristics has been studied in various developmental aspects, however, it has not yet been documented in detail for behavioral traits. Behavioral studies of cloned animals have failed to show clear inter-individual differences associated with the cloning process. Preliminary results showed that clones favor each other's company. Preferential social interactions were observed among cloned heifers from the same donor in a mixed herd that also included cloned heifers and control heifers produced by artificial insemination (AI). These results suggest behavioral differences between cloned and non-cloned animals and similarities between clones from the same donor. The aim of the present study was to replicate and to extend these previous results and to study behavioral and cognitive mechanisms of this preferential grouping. We studied a group composed of five cloned heifers derived from the same donor cow, two cloned heifers derived from another donor cow, and AI heifers. Cloned heifers from the same donor were more spatially associated and interacted more between themselves than with heifers derived from another donor or with the AI individuals. This pattern indicates a possible kin discrimination in clones. To study this process, we performed an experiment (using an instrumental conditioning procedure with food reward) of visual discrimination between images of heads of familiar heifers, either related to the subjects or not. The results showed that all subjects (AI and cloned heifers) discriminated between images of familiar cloned heifers produced from the same donor and images of familiar unrelated heifers. Cattle discriminated well between images and used morphological similarities characteristic of cloned related heifers. Our results suggest similar cognitive capacities of kin and non kin discrimination in AI and cloned animals. Kinship may be a common factor in determining the social grouping within a herd.

Keywords: Cattle; Somatic cell nuclear transfer; Social behavior; Kin discrimination; Instrumental conditioning; 2D-images of head

1. Introduction

Mammalian cloning using somatic cells was first demonstrated in sheep in 1997 [1] and since then has been extended to a number of other mammals including other ruminant species (e.g. cattle [2] and goat [3]). The success in generating cloned animals has induced the development of new areas of research such as genetic

* Corresponding author. Tel.: +33 1 49 40 32 59; fax: +33 1 49 40 39 75.
E-mail address: marjoriecoulon82@yahoo.fr (M. Coulon).

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reprogramming, embryonic development, therapeutic cloning of human cells, tissue or organ replacement, and possible agricultural applications including reproductive cloning of farm animals [4–6].

In this reproductive context, it is critical to determine the consequences of the technique of cloning in resulting offspring [7], because according to physiological measures, clones are not always phenotypically identical to their somatic cell donors. Reports of telomere length [8], genomic imprinting [9], DNA methylation [10], and mitochondrial DNA genotype [11] suggest that cloned animals may harbor genetic aberrations that could have detrimental future consequences. In addition, behavioral studies are needed in order to ensure the well being of cloned animals and to identify possible effects of cloning. The physiological differences found in some individuals might have consequences on their behavior. Unfortunately, behavioral studies in cloned animals are rare. In addition they do not seem to reveal behavioral differences between cloned and non cloned animals. However, interestingly, these studies suggest that clones recognize each other. For example, in a previous study on social behavior of domestic cattle, no behavioral differences were observed between cloned heifers of various origins and control heifers obtained by artificial insemination (AI) [12]. In this group, social dominance appeared to be linked to body weight and age rather than to a cloning effect [12]. By contrast, studies of social behavior of cloned cows, living in a mixed group of cloned and AI animals, showed preferential interactions among cloned heifers produced from various donors [12] and among cloned heifers produced from the same donor [13]. It is possible that the mechanisms of these preferential groupings were, in fact, cases of kin discrimination, partially mediated by head morphological similarity. Kin discrimination refers to the differences in behavioral responses that an individual shows toward its kin as compared to non-kin animals [14]. Kin association and discrimination occur in various invertebrate and vertebrate species [15,16]. Social relations based on genetic relatedness generally provide adaptive benefits [17–19].

In order to test the hypothesis of the existence of kin discrimination by heifers, we used an instrumental conditioning procedure [20,21] to study their capacity of visual discrimination between head images of cloned cows (produced from the same donor) and unrelated cows. We have previously used this method to show that cattle possess cognitive capacities of visual species discrimination and individual recognition [20,21].

Table 1
Characteristics of heifers involved in the behavioral study (experience 1) and in the visual discrimination study (subjects indexed with *: experience 2).

N° heifers	Origin	Dam	Sire	Age (months) at observational period
B1	Clone B	M1	S1	18.5
B2	Clone B	M4	S1	17
D1*	Clone D	M10	S5	14
D2*	Clone D	M14	S5	10.5
D3*	Clone D	M15	S5	10.5
D4*	Clone D	M15	S5	10.5
D5*	Clone D	M19	S5	10
1	A.I.	M2	S2	18
2	A.I.	M3	S3	18
3	A.I.	M5	S4	16.5
4	A.I.	M6	S5	16.5
5	A.I.	M7	S6	16
6	A.I.	M8	S7	16
7*	A.I.	M9	S4	14
8	A.I.	M11	S8	11
9*	A.I.	M12	S3	11
10*	A.I.	M13	S9	10.5
11*	A.I.	M16	S10	10.5
12*	A.I.	M17	S6	10
13	A.I.	M18	S5	10
14*	A.I.	M20	S6	10
15	A.I.	M21	S11	10
16	A.I.	M22	S11	9
17*	A.I.	M23	S12	8

The aims of the present study were a) to verify if cloned animals from the same donor tend to interact more between themselves and to be more spatially associated together than with other heifers (cloned ones derived from another donor and AI heifers) b) to determine if heifers discriminate between images of head from familiar unrelated or familiar kin related individuals, and c) to compare cognitive capacities of cloned and AI subjects.

2. Materials and methods

2.1. Animals and housing

We studied a social group of 24 Prim'Holstein heifers (*Bos taurus*) aged from 8 to 18.5 months at the start of the experiment (Table 1). Seven heifers were produced by somatic cloning and the remaining 17 heifers were produced by AI from 10 different Holstein bulls of 17 different Prim'Holstein dams (Table 1). The seven cloned heifers were produced from adult somatic cells of two different genotypes (B and D, Table 1). All animals were born and raised at the INRA (National Institute of Agronomical Research) experimental farm T1 in Bressonvillers (France). Before six months of age, all animals lived in individual stalls. At six months, the animals were grouped together in the same loose house system. The surface of the house system was extended as soon as the heifers were grouped. This stall (11 × 18 m) was composed of an area with straw (8 × 18 m) and an area (3 × 18 m, with 24 feeding yokes) with free access to unrestricted food. In the first part of this study, all 24 heifers were observed and 12 heifers (five cloned heifers produced from the donor D and seven AI heifers) were tested in second experimental part (subjects indexed with * in Table 1).

2.2. Behavioral study (experiment 1)

2.2.1. Social behaviors

Our procedure was similar to that used in a previous study [12]. Observation sessions occurred from 4 p.m. to 8 p.m., four times a week, for 5 weeks. This observation time has been chosen because it was the quieter time of the day according to the farm activities. Each heifer was observed for two 3-min periods (focal animal sampling) per session. The minimal time interval between two successive periods was two hours and the order of observed individuals was randomly assigned each day. Types and frequency of social behaviors were recorded as well as the identity of the recipient. The following social behaviors were recorded: agonistic behaviors with offensive behaviors (offensive approaches with threats, butts and fights) and defensive behaviors (spontaneous withdrawals, escapes) and non-agonistic behaviors (non agonistic approaches with sniffing, licking, rubbing, head supports on the back of another animal). The number of behaviors was recorded for each subject and for each dyadic interaction in a matrix of observed individuals.

The position of each heifer was noted every half hour between 4 p.m. and 8 p.m., eight times per day as follow: lying down in the area of straw, free in the area of straw, free in the area of feeding yokes and eating at a feeding yoke and the identity of the nearest neighbor(s) (less than one meter). For each position of one observed individual all animals placed less than one meter from the closest part of its body were recorded in a similarity matrix of observed individuals.

2.2.2. Statistical analyses

Data were analyzed using the statistical method DISTATIS, which is a three-way generalization of classical multidimensional scaling (MDS) [22–25]. The DISTATIS method was used to study the relationship between individuals based on their three behavioral types: offensive, defensive and non-agonistic behav-

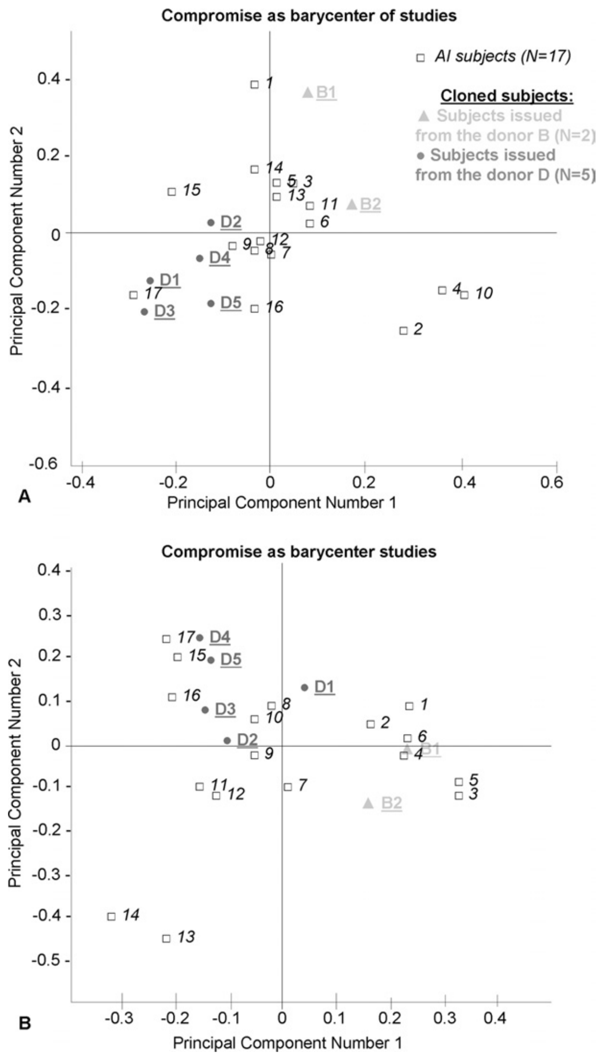

Fig. 1. Plots of heifers living in a stable group and characterized by their behaviors (agonistic offensive, agonistic defensive and non-agonistic behaviors) (A), and their spatial distribution in the free stall (B). The closer two individuals are in the plane the more they interacted together (A) and the more they were spatially associated (B). The group was composed of 24 heifers with 17 heifers produced by artificial insemination (□ *Individuals 1 to 17*) and 7 heifers issued from somatic cloning of a donor D for five of them (● **D1 to D5**) and of a donor B for two others (**Δ B1 to B2**). AQ: 1

iors. Each type of behavior was presented in an Individual × Individual matrix, in which the data were the total number of dyadic interactions between individuals. Three matrices were constructed and from these matrices we created a new matrix of mean distances from which we derived a map (Fig. 1A) representing F1 the distances between individuals.

The DISTATIS method was also used to study the physical associations (which were used as a criterion of relationship between individuals) based on their four positions and activities in the stall: lying down in the area of straw, free in the area of straw, free in the area of feeding yokes, and eating at a feeding yoke. As previously, Individual × Individual matrices were constructed with the data being the total number of two individuals observed together in each position. With the four obtained matrices we created a matrix of mean distances and a map representing the distances (Fig. 1B) between individuals described by this matrix.

In order to confirm that the pattern of association between heifers was systematic, we used a bootstrap procedure to compute confidence intervals for the three groups of heifers. We decided to use bootstrap procedure because the analytical distribution of the confidence interval is not known and because the bootstrap method builds non-parametric maximum likelihood confidence interval [26,27]. In order to compute these bootstrapped confidence intervals, we sampled with F2 replacement observation (i.e. cows, Fig. 2A: behaviors, Fig. 2B: physical associations) from each of the three groups and computed a mean for these three bootstrapped samples. The distribution of these means was then trimmed to keep 95% of the means, and these sets of means were then plotted as confidence interval. When the confidence intervals of two groups do not overlap, these groups are significantly different (see [25] for more details on this procedure).

The first dimension explained only 11% of the variance (against an expected value of 4% by chance). This small proportion of explained variance is a consequence of using presence/absence (i.e. 0/1) data [22,23] because most of the data values are null. In order to check that the first dimension is not an artifact, we used a permutation test and found that the percentage of variance extracted by the first dimension was highly significant ($P < .0001$).

2.3. Visual discrimination study (experiment 2)

2.3.1. Stimuli

The stimuli consisted of 20 prints (50 × 42 cm) of digitized color pictures of heads of familiar Prim'Holstein heifers (familiar = living in the same social group as the subject). The pictures were approximately in real size and were taken with a same uniform background [20,21]. The 20 prints represented the heads of heifers from different viewing angles (frontal view = face, right and left profile views, right and left ¾ frontal views, right and left ¾ back views). The task was to discriminate between two series of 10 stimuli, one series representing three different cloned heifers produced from the donor D and the other series, three different AI heifers. All of the images used in this experiment were representations of familiar individuals. The area ratio of white and dark spots areas (W/D) was similar in stimuli from the two categories (cloned heifers stimuli vs. AI heifers stimuli, respectively W/D = 0.24 and W/D = 0.33: $F_{1,18} = 0.81$, $P = 0.31$). The ratio was similar within the cloned heifers stimuli (W/D = 0.27, 0.28, and 0.47: $F_{2,7} = 0.51$, $P = 0.73$) but significantly different within the AI heifers stimuli (W/D = 0.25, 0.05, and 0.53: $F_{2,7} = 41$, $P < 0.001$).

2.3.2. Experimental procedure

We use the same experimental procedure and apparatus as in our previous study of visual discrimination

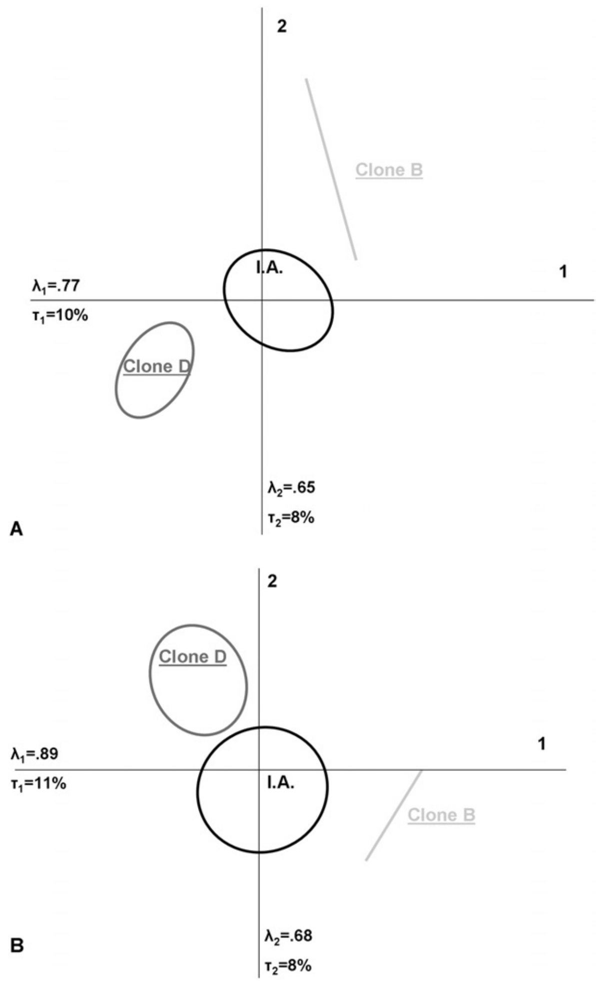

Fig. 2. Confidence intervals for the three groups of heifers (cloned heifers B, cloned heifers D and AI heifers) computed with a bootstrap procedure. The confidence intervals of each group do not overlap, so these groups are significantly different in their social interaction (A) and physical interaction (B). The variance explained by each dimension is denoted λ and its percentage of explained variance is denoted τ.

of the species [20]. Briefly, this procedure was an instrumental conditioning based on a simultaneous discrimination S+ / S− stimuli, using food reward. Heifers were tested individually in a familiar test pen (6 × 11 m). The subject walked to a guillotine gate at the end of a lane made between rows of straw bundles. The heifer could see the two images placed at its eye level. For each pair of stimuli, one stimulus was consistently associated with a reward, S+. After the heifer has looked at both stimuli, the experimenter lifted the gate. The heifer could then walk towards the chosen image. For a correct choice, the subject had to push an opaque panel in order to access the reward. In case of an incorrect choice, this panel was blocked. To avoid any olfactory bias, a reward was always placed behind each panel. The left/right position of the rewarded stimulus was randomly balanced across trials.

The criterion of success was set as at least 80% of good responses per session of 10 trials, during 2 consecutive sessions. The experiment included a training phase and a generalization phase.

2.3.2.1. Training. The same pair of stimuli was presented at each trial and consisted of the frontal view of one cloned heifers and the frontal view of one AI heifer. Stimuli, representing cloned heifers, were rewarded for two cloned and five AI subjects and stimuli, corresponding to AI heifers, were rewarded for three cloned and two AI subjects.

2.3.2.2. Generalization phase. After reaching the learning criterion in training, animals were tested in a generalization test for which all the 20 images were used. The pair of stimuli—one stimulus of a cloned heifer and one stimulus of an AI heifer—changed at every trial. Each stimuli of this pair corresponded to one of the various views: frontal, profile, ¾ frontal and ¾ back views. The stimuli of the pair could be in the same view or in different views. We stopped testing an animal if it did not succeed in 20 sessions (for a total of 200 trials). The same groups of stimuli (cloned or AI heifers) as during training were rewarded during the test phase.

2.3.2.3. Control phase. The control experiment included only the generalization phase between two series of new stimuli of three familiar heifers. Each series of three heifers was composed of one cloned heifer from the clone D and two other AI heifers. The animals were still rewarded for choosing the image of three heifers among the six. The procedure of test and the criterion were the same as in the experimental phase. The animals had to discriminate between pairs of stimuli of 1) AI heifers, 2) cloned heifers from the same donor and 3) pairs of stimuli composed of AI and cloned heifers. For each of these three kinds of pairs of stimuli, we calculated the error rate corresponding to the number of errors divided by the number of successes.

2.3.3. Statistical analyses

Discrimination performance was measured by the number of sessions needed to achieve successful discrimination (as define above). We used a Mann Whitney test to confirm that there was no difference of performance between the types of stimuli rewarded. We analyzed the success of the experiment (experimental vs. control), the effect of cloning (cloned heifers tested vs. AI heifers) and their interactions with a repeated measurement analysis of variance (ANOVA). We used the same test to study in the control phase the effect of the kind of pair of stimuli, the effect of cloning and their interaction on the error rate. The parameters (error rate) for which the standardized residuals did not meet the assumption of normality on a QQ plot and the homogeneity of residuals variance were log transformed prior to the statistical tests.

Statistical analyses were carried out using the SAS statistical package (Version 8.1., SAS Institute, Cary, North Carolina) and the JMP statistical package (Version 8.0., SAS Institute). Significance was set at the .05 level.

2.4. Ethical note

Animal care and all procedures were completed in accordance with the authorization 93-031 delivered by the "Préfecture de la Seine-Saint-Denis, direction départementale des services vétérinaires," and the authorization "B91 332 101" of the French Ministry of Agriculture and the EU directives. The protocol, registered as "protocol 06-002," was approved by the Regional Ethical Committee of Paris-Sud.

3. Results and discussion

3.1. Behavioral study: Social interactions and physical associations between heifers (experiment 1)

There was no strict segregation between cloned and AI heifers (Fig. 1). However, the cloned heifers from a particular genotype (donor B or D) appeared to be more associated and interacting between them than with others (Fig. 1A, 1B). The boostrapped analysis showed that the confidence intervals of the two groups of clones did not overlap and this pattern confirms that social interactions (Fig. 2A) and physical associations (Fig. 2B) among the three groups (cloned heifers from the donor B, cloned heifers from the donor D and AI heifers) were significantly higher than between the three groups. This organization in subgroups of genetically related individuals (cloned heifers from the same donor) with preferential interactions suggests the existence of some capacities of kin discrimination or recognition. These results confirm and extend those of two previous studies showing respectively associations between four cloned cows derived from a unique donor [13], and more social interactions between cloned heifers than with AI heifers in a mixed group [12]. We did not observe preferential social interactions or physical associations among related AI heifers sharing the same bull (e.g. heifers #2 and #9 or heifers #5 and #12, Fig. 1). Preferential associations and interactions between genetically related animals were also observed in other species [28,29]. In contrast, previous studies in twin calves related the preferential interactions between kin more to familiarity than to genetic relatedness [30] and Murphey [31] showed in free-ranging cattle in the Sonoran Desert of Mexico, that familiarity is more important than shared genes in the establishment of aggregations. In our study, the familiarity was the same for all the animals at the time of the composition of the group and independent of their genetic proximity. However the older heifers spent more time together in the social group than the younger heifers. The age of the heifers may have had an effect on their physical associations. The repartition of the heifers follows the first axis according to the age of the individuals (Fig. 1B). The older heifers are present on the right of the map and the younger on the left. The interactions between heifers appeared more age-independent (for example heifers #1 and #2 were close in age but far away in their interactions, Fig. 1A). This result is congruent with our previous study [12] showing the role of agonistic and non agonistic social behaviors in the subgroup formation. Our new results suggest for the first time that the social organization in a mixed group of familiar heifers may depend also on the genetic proximity between individuals.

What makes possible this type of association based on kinship? Cloned heifers produced from a same donor cow were probably more similar (morphological traits, odor, behavior, etc.) between them than with other cloned or AI heifers and social preferences may be based on physical (visual, acoustic, or chemical cues) or other behavioral similarities. We indeed observed more exploratory behaviors in cloned heifers [12] and Savage et al [13] reported more agonistic behaviors in clones. Along the same lines, Murphey [32] showed in the social aggregation of three cattle breeds that recently weaned calves formed phenotypic groups correlated with their coat color. According to Murphey [32], in the absence of familiar individuals, cattle may use familiar phenotypes in establishing social preferences and cohesive herds. His conclusion is based on Schloeth [33] who noted that vision dominates other sensory modalities in the social behavior of free-ranging cattle or that color is a specific cue in aggregation of cattle. In our study, it appears that heifers discriminate kin from non-kin and that this discrimination process could be based on morphological similarities and behavioral affinity. The heads of cloned heifers produced from the cloned D are indeed more similar in their ratio of back and white spots compared to heads of individual AI heifers that were significantly different in their ratio of black and white spots. We tested this hypothesis with the study of kin visual discrimination in the second part of this study.

3.2. *Visual discrimination study (experiment 2)*

The results showed that subjects discriminated between related and unrelated familiar individuals whatever the image (S = stimulus) rewarded (S+ vs. S−, U = 23.5, NS), more quickly than in the control task (Generalization phase vs. Control phase, $F_{1,23} = 5.82$, $P = 0.036$, $N = 12$, Fig. 3). Both cloned and control heifers were able to perform the task of discrimination. The performances of cloned heifers did not differ significantly from those of AI heifers suggesting that they may have similar cognitive capacities (Cloned vs. AI, F3

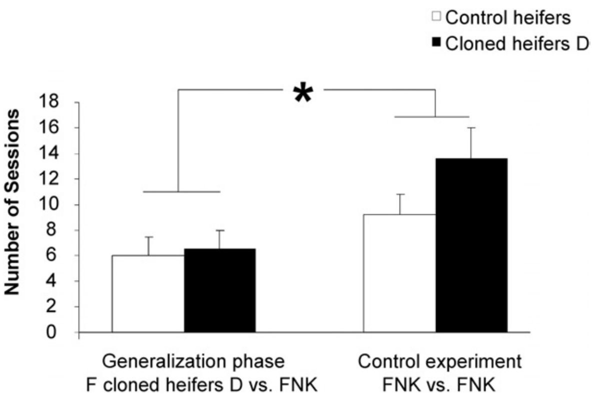

Fig. 3. Discrimination between images of three familiar (F) cloned heifers, produced from the donor cow D, and three familiar non-kin heifers (FNK). Number of sessions (Mean + SEM) to reach the criterion level during the generalization phase and the control phase (FNK vs. FNK). Number of subjects: $N_{cloned\ D\ heifers} = 5$, $N_{AI\ heifers} = 7$, * = P < .05.

$F_{1,23} = 2.61$, $P = 0.14$, $N_{cloned} = 5$, $N_{AI} = 7$) and that there was no effect linked to cloning (i.e., heritability of the donor D's cognitive characteristics as a consequence of the technique of cloning in resulting offspring). We did not observe an effect of interaction between discrimination tasks and categories of subjects (AI/Cloned heifers × tasks, $F_{1,23} = 0.93$, $P = -0.36$). At this stage we do not know if heifers discriminated between related individuals and others due to their familiarization with their conspecifics before the experiment or due to visual stimuli characteristics during this experiment. In the first case, the heifers probably learned some specific characteristics of their conspecifics during social interactions. In the second case, the similarities between kin head stimuli (i.e. cloned heifers from the same donor) favored the discrimination process [34]. Such an effect of familiarization in the discrimination between conspecifics has been shown in several previous studies. For example, Stookey and Gonyou [35] showed that discrimination among young piglets appears to be based on familiarity through rearing association and did not seem to involve genetic relatedness. Along the same lines, Dasser [36] showed that Java monkeys correctly match views of familiar offspring to their respective mother using recent slides. In our study, it appears that cattle can discriminate between familiar kin and non-kin using only visual stimuli provided by a picture of the head. As the cloned heifers produced from the same donor are more morphologically similar between them than with other individual heifers, we can suppose that it will be more difficult to discriminate within a category of individual cloned heifers. In the control phase, the error rate for the pair of stimuli composed of two cloned heifers were numerically higher (43%) than the error rates for the other kind of pair of stimuli (two AI heifers to discriminate or the discrimination between one AI heifer and one cloned heifer, 37% each). However the difference in the error rates was not significantly different ($F_{2,31} = 0.42$, $P = 0.66$), the error rates were not different according to the category of subjects tested (cloned heifers vs. AI heifers, $F_{1,31} = 1.41$, $P = 0.24$) and there was no interaction between the category of subjects tested and the category of pair of stimuli ($F_{2,31} = 0.85$, $P = 0.44$). This last result shows that discriminating within cloned individual heifers produced from the same donor looks more difficult than discriminating between less related subjects but it seems that the heifers can perform it and individually recognize very closely related individuals.

4. Conclusions

Our study showed that in the natural context of a stable social group, genetically close heifers interact more among themselves and are spatially closer than they are to non-kin heifers. In domestic cattle, the structure of the herd can be organized in subgroups of familiar animals [31] and our study showed that this type of social organization could be based also on genetic relatedness. The existence of subgroups of clones issued from the same donor suggests that kin discrimination may occur as a basis of grouping. In an experimental situation using 2D-head images, we have demonstrated that both cloned and AI heifers could discriminate kin-related conspecifics using only visual cues from the head. Cloned and AI heifers exhibited similar patterns of behavioral and cognitive performances as one another. These results call for additional studies of cognitive capacity and kin recognition in domestic cattle.

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