Applied Animal Behaviour Science 141 (2012) 20–24

Contents lists available at SciVerse ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim

# Effect of genetic homogeneity on behavioural variability in an object recognition test in cloned Göttingen minipigs

Lene Vammen Søndergaard[a,b,*], Mette S. Herskin[b], Jan Ladewig[c], Ida Elisabeth Holm[d,e], Frederik Dagnæs-Hansen[a]

[a] Department of Biomedicine, Aarhus University, Wilhelm Meyers Allé 4, DK-8000, Aarhus C, Denmark
[b] Department of Animal Science, Aarhus University, Blichers Allé 20, Postboks 50, DK-8830 Tjele, Denmark
[c] Department of Large Animal Sciences, University of Copenhagen, Grønnegårdsvej 8, DK-1870 Frederiksberg C, Denmark
[d] Laboratory for Experimental Neuropathology, Department of Pathology, Randers Hospital, Skovlyvej 1, DK-8930 Randers NØ, Denmark
[e] Institute for Clinical Medicine, Aarhus University, DK-8000 Aarhus, Denmark

ARTICLE INFO

*Article history:*
Accepted 9 July 2012
Available online 9 August 2012

*Keywords:*
Cloning
Variability
Swine
Behaviour
Cognitive test
Memory

ABSTRACT

The number of animals used in research should be limited as much as possible. Among cloned animals, genetic variation is minimal and to the extent that behaviour is genetically determined inter-individual variability is expected to be higher among naturally bred animals. However, the cloning procedure per se might affect the resultant phenotype leading to phenotypic variations independent of the genetic background. Recently, cloned Göttingen minipigs carrying a mutation for Alzheimer's disease have been produced. In order to document the development of Alzheimer's disease symptoms, these pigs were subjected to a behavioural test of memory, the spontaneous object recognition test, from an early age. At ages 1 and 2 years no evidence of memory decline was found, yet the data showed striking behavioural variability among the cloned groups. The aim of the present study was to investigate effects of genetic homogeneity on variability of cloned minipigs compared with non-cloned controls regarding behavioural variables in a cognitive test, namely the spontaneous object recognition test.

Significant differences in the variability between the cloned and control pigs were found in five out of 24 behavioural variables. The clones showed lower variability for four of the measures, whereas the variability was increased for one variable. No significant difference between the cloned and the control group was found for the remaining 19 variables (79%). In 14 of these, the standard deviation was numerically greater for the control group compared to the cloned group, indicating that variation may be less in cloned animals, but not demonstrable with the small group size of the present study ($n = 6$ for each of the two groups tested).

Overall, this study failed to show unambiguously that variability in these behavioural variables in cloned minipigs is less than in naturally bred control subjects and therefore does not directly support the hypothesis that cloning may be used to replicate animals in order to reduce group size in experimentation animals.



## 1. Introduction

Cloning technologies are being used increasingly to produce disease models, i.e. animals designed to express, either at the genotypic of phenotypic level, a certain human disease (Vajta and Gjerris, 2006). According to guiding

* Corresponding author at: Department of Biomedicine, Aarhus University, Wilhelm Meyers Allé 4, DK-8000, Aarhus C, Denmark.
Tel.: +45 8715 7937; fax: +45 89 99 11 66.
*E-mail address:* Lenev.sondergaard@agrsci.dk (L.V. Søndergaard).

0168-1591/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.applanim.2012.07.006

principles for the use of animals in research, the "three Rs" (Russell and Burch, 1959), the number of experimental animals used should be limited as much as possible. In clones, genetic variation is minimal, leaving only environmental influence, for which reason inter-individual variability is expected to be less than among naturally bred animals. However, the cloning procedure per se might affect the phenotype and cloning has been observed to affect the expression of traits in cloned mice (e.g. Humpherys et al., 2002) and pigs (e.g. Carter et al., 2002; Schmidt et al., 2010). To date, only limited data on behavioural variability in pigs has been published (Archer et al., 2003), showing that groups of genetically identical clones (group size of 4–5 animals) do not show increased behavioural consistency as compared to naturally bred animals.

Recently, cloned, Göttingen minipigs carrying a mutation for Alzheimer's disease have been produced (Kragh et al., 2009). These pigs were healthy with a normal phenotype and concordance was expected in gene expression and development of disease phenotype. Since age-dependent memory impairment is a prominent clinical symptom in Alzheimer's disease, these pigs were subjected to a validated behavioural test of memory, the spontaneous object recognition test (Moustgaard et al., 2002; Kornum et al., 2007), from an early age. At ages 1 and 2 years, no behavioural evidence of memory decline was found (Søndergaard et al., 2012) and the brains were normal with no evidence of beta-amyloid deposits or neurofibrillary tangles (Holm, personal communication). Interestingly, the data showed striking behavioural variability among the cloned groups, and this finding was further examined in the present study where the variability of cloned minipigs was compared to that of non-cloned minipigs regarding behavioural responses in a cognitive test. We hypothesised that the genetic homogeneity of clones would result in significantly less variability than among naturally bred controls.

## 2. Materials and methods

### 2.1. Animals and training

Twelve female Göttingen minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were used at two ages (1 and 2 years). Six of the animals were clones produced by handmade cloning, carried the APPsw mutation for Alzheimer's disease (Kragh et al., 2009) and were born at the facility by the same surrogate sow. The control group consisted of six age-matched, non-cloned, non-transgenic minipigs which were pair wise littermates, and aged 10 months at arrival. The pigs were kept in one room in pairs (three pairs of transgenic minipigs and three pairs of controls) in pens of 155 cm × 150 cm. Visual and some tactile communication was possible between animals in neighbouring pens through pen separating bars and over the pen walls (75 cm high). The animals were fed restricted twice a day (7.30 a.m. and 2.30 p.m.) with standard minipig pellets according to producer's recommendation (Special Diets Services, Essex, England), and had unlimited access to water from nipple drinkers. Room temperature was kept at 20 °C and circadian rhythm was insured by electrical light from 7.30 a.m. to 2.30 p.m. Animals were kept on daily renewed wood chips bedding provided after morning feeding and occasionally provided artificial environmental enrichment (sisal robe, wood bricks) to accustom the animals to novel objects. Before the experimental phase the pigs were habituated to the experimenter and in-house transport in a carrier over at least three weeks. For this procedure, motivational factor was rewarding of appropriate behaviour with 0.9 g chocolate pellets (Mars Scandinavia, Copenhagen, DK). Trials and handling were conducted between 8.30 a.m. and 2.30 p.m.

All animal experiments were performed in accordance with the European Communities Council Resolves of 24 November 1986 (86/609/Ecc) and approved by the Danish Experimental Animal Inspectorate (journal number 2006/561-1156).

### 2.2. Experimental setup

In a testing arena (350 cm × 300 cm) in a separate experimental room, test objects were mounted on each side of the wall containing the door to the arena, 35 cm from the floor and 50 cm from the corners.

The objects were different plastic household items, and a set of objects was defined as two objects of similar colour and approximate size, but different shape. Objects were chosen on the basis of a previous evaluation of object bias (Kornum et al., 2007). The testing arena and the objects were cleaned with hot water between each separate test phase to minimise olfactory traces. During all test sessions the experimenter stayed outside the experimental room. The pigs were habituated to the empty arena for 5, 10 and 20 min on three separate days. This was followed by a single habituation session lasting 10 min in which two identical objects were presented. Testing began two days after the final habituation.

Prior to each test, the pig stayed 5 min in the testing arena to habituate to the situation. The pig was then removed and two identical objects (A1 and A2) were placed on the wall. The pig was let back in the arena and allowed 10 min of free exploration (sample phase), after which the pig was transported back to the home-pen. The test phase was initiated after a fixed inter-phase interval of 10 min. A novel object (B) and a familiar object (termed A3, i.e. A1 or A2 randomly chosen and rinsed in hot water) were placed in the arena and the pig allowed 10 min of spontaneous exploration. The testing order of the pigs was randomised, yet three cloned and three control subjects were tested each day. The designated familiar and novel object for each set was randomised. The testing arena was equipped with a video camera (Panasonic CCTV Camera Model No. WV-BP330/G, Osaka, Japan) to record all trials on a digital hard disc recorder (MSH-Video, Riga, Latvia).

### 2.3. Data acquisition

All test trials were analysed manually by a blinded observer using the MSH Video software (MSH-Video, Riga, Latvia). Explorative behaviour directed at objects was defined as the pig having physical snout contact with the object or placing the snout directly over the object. Using

**Table 1**
Variables used to assess memory of cloned and control Göttingen minipigs in the spontaneous object recognition test (from Ennaceur and Delacour, 1988).

| Variable | Description |
|---|---|
| Exploration 1 (E1) | Duration of time spent exploring the objects A1 and A2 |
| Exploration 2 (E2) | Duration of time spent exploring the objects A3 and B |
| Habituation 1 (H1) | Habituation to the test situation: E1 – E2 |
| Habituation 2 (H2) | Habituation to the familiar object: (E1/2) – A3 |
| Discrimination 1 (D1) | The difference between time spent exploring the novel and familiar objects: B – A3 |
| Discrimination 2 (D2) | D1 normalised to total exploration in the test phase: D1/E2 |

duration of exploration of the objects during the sample phase (directed at A1 and A2) and during the test phase (directed at A3 and B), the variables in Table 1 were calculated, based on the original definitions by Ennaceur and Delacour (1988). Similar variables were computed for latency to initial contact.

### 2.4. Data analysis

In order to test if variability among clones differed from naturally bred controls, one-tailed *F*-tests (Glantz, 2001) were used to compare the variance of the two groups within each age. Normality was checked using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA), and a logarithm transformation of data was performed in case of non-normalised data. Data are presented as mean ± standard deviation (SD) and a level of significance of 0.05 was applied.

## 3. Results and discussion

This study is among the first studies of the effect of cloning-induced genetic homogeneity on behavioural measures in pigs. Genetics plays a significant role in controlling specific behaviour, and the clearest examples of the genetic basis of behaviour are shown in mice, since detailed genetic maps, highly polymorphic markers and inbred strains are available in this species (Búcan and Abel, 2002). We examined effects of cloning on the behaviour of Göttingen minipigs in a cognitive test, hypothesising that the inter-individual variability among clones would be less than among naturally bred animals.

Significant differences between the two groups of animals were found for five out of the 24 variables presented in Figs. 1 and 2. Here, the cloned animals showed lower variability for four measures (H2 (latency) at age 1 year and E2, H1 and D1 (duration) at age 2 years), whereas the variability was increased for one variable (H2 (duration) at age 1 year). Consequently, no significant difference between the cloned and the control group was found for the remaining 19 variables (79%). In 14 of these the standard deviation was numerically greater for the control group as

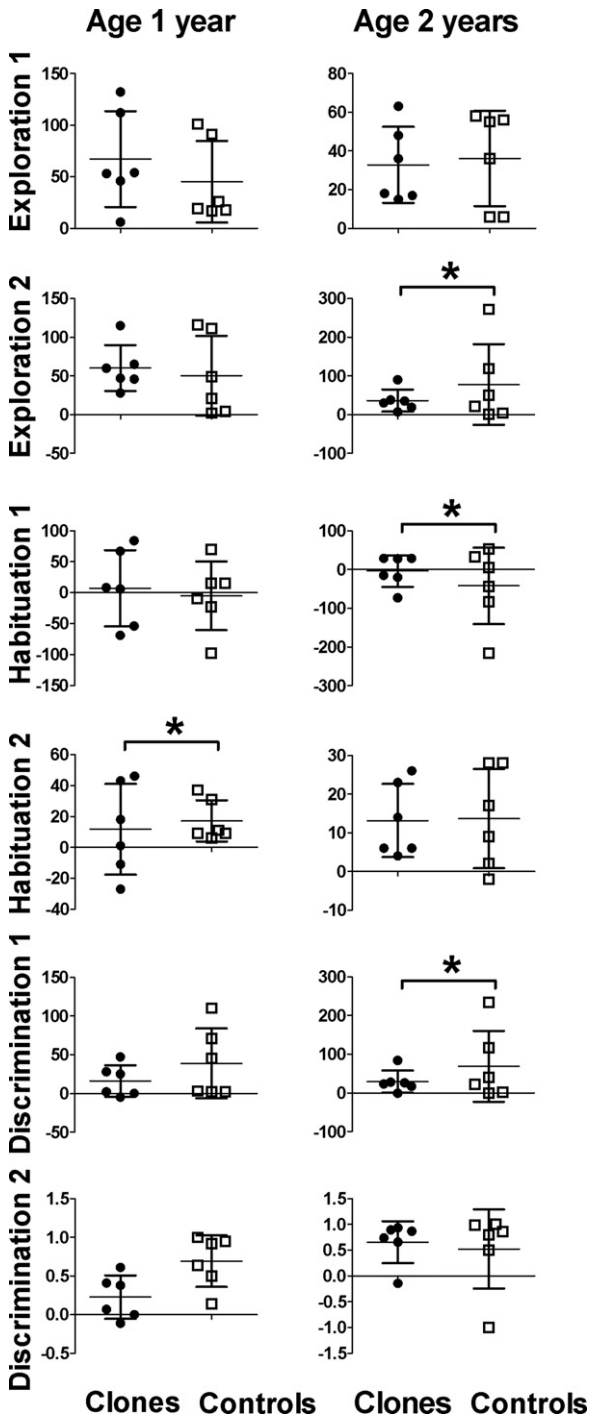


**Fig. 1.** Durations of different measures of exploration from the spontaneous object recognition test (mean ± SD) for cloned and control minipigs. *Y*-axis denotes duration (s) of contact during a 10 min test. Each row shows a variable (row 1: Exploration 1; row 2: Exploration 2; row 3: Habituation 1; row 4: Habituation 2; row 5: Discrimination 1; row 6: Discrimination 2). Each column shows different ages of the minipigs (column 1: age 1 year, column 2: age 2 years). Asterisks: significant differences in one-tailed *F*-test between cloned and control minipigs ($P < 0.05$).

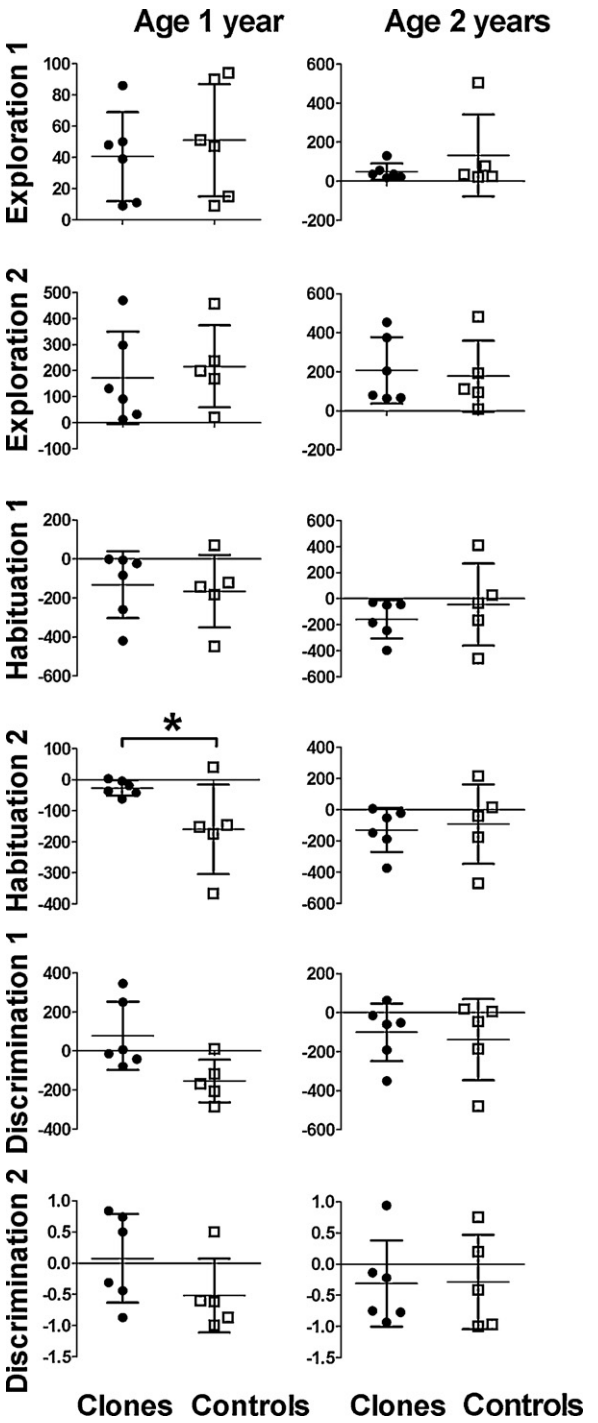


**Fig. 2.** Different measures of latencies to initial contact to object in the spontaneous object recognition test (mean ± SD) for cloned and control minipigs. *Y*-axis denotes latency (s) to initial contact to object during a 10 min test. Each row presents a variable (row 1: Exploration 1; row 2: Exploration 2; row 3: Habituation 1; row 4: Habituation 2; row 5: Discrimination 1; row 6: Discrimination 2). Each column shows different ages of the minipigs (column 1: age 1 year; column 2: age 2 years). Asterisks: significant differences in one-tailed *F*-test between cloned and control minipigs ($P < 0.05$).

compared with the cloned group, suggesting that variation may be less in cloned animals, but not demonstrable with the small group size of the present study. The test failed to show unambiguously that behavioural variability in exploration and object discrimination in cloned minipigs is less than in naturally bred control subjects. Because cloning of animals from somatic cells involves epigenetic reprogramming of the genome it is conceivable that imperfection of this process contributes to the observed phenotypic variation (Reik, 2007). Such questions should be addressed before any decision is made as to whether cloning is the right method to produce animals in order to reduce experimental group size. Detailed investigation of this warrants further research including larger and more standardised experimental groups.

The findings of the present study support the conclusion by Archer et al. (2003), stating that groups of genetically identical clones (4–5 animals per group) do not show increased behavioural consistency as compared to naturally bred animals. Archer et al. (2003) investigated effects of genetic homogeneity on behavioural variation in pigs using different standardized tests. In either of the tests, however, the authors were able to show consistent difference in the variability between cloned and non-cloned animals leading to the conclusion that the variation in behaviour among genetically identical clones does not seem to be smaller than among litters of naturally bred pigs.

It should be kept in mind that the animals in the present study were not only cloned, but had also been exposed to genetic modification in terms of transgenesis. No effects of the APPsw transgene on the memory of the animals could, however, be demonstrated using the test described. This may not be surprising considering the temporal disease progression in human AD patients. In the human brain, APP-mutations cause complete AD phenotype including plaques, tangles, neuron loss and progressive cognitive impairment. Memory decline typically becomes clinically evident relatively late in life, and earliest at the age of 40–50 years (Ringman, 2005). Drawing analogy from the temporal disease development of human AD patients to pigs is controversial and not straightforward. The assumed life-span of pigs may be around 15–20 years of age; however this estimation is difficult to substantiate, since the vast majority of pigs are euthanized before reaching adulthood. One reliable comparable measurement might be the brain development; in humans, brain development ceases around the age of 18 years, whereas the corresponding porcine age is approximately 18 months (Swindle M.M. (Vet. Med. Biomed. Sci., Texas A&M Univ., Texas), personal communication). Consequently, it may not be surprising that memory decline was not evident in the present study using pigs of 1 and 2 years of age. Consistent with this result, neuropathological examination using immunohistochemical staining for Aβ, tau protein and ubiquitin showed normal histology without deposition of Aβ or tau protein in one 2-year-old transgenic minipig clone (data not shown). In conclusion, the variability among the clones was numerically less than the controls for more than 70% of the included variables, but only statistically significant for a subset of variables (17%).

Overall, this study failed to show unambiguously that variability in the included behavioural variables in cloned minipigs is less than in naturally bred control subjects and therefore does not directly support the hypothesis that cloning may be used to replicate animals in order to reduce group size in experimentation animals. In order to investigate this in more detail, further research is warranted with larger and more standardised experimental groups.

## Acknowledgements

This work was supported by the Danish Agency for Science Technology and Innovation [grant number 274-05-0197], and supported by Institute of Clinical Medicine, Aarhus University. The authors thank Birgitte R. Kornum for invaluable assistance through all phases of the study.

## References

Archer, G.S., Friend, T.H., Piedrahita, J., Nevill, C.H., Walker, S., 2003. Behavioral variation among cloned pigs. Appl. Anim. Behav. Sci. 82, 151–161.

Búcan, M., Abel, T., 2002. The mouse: genetics meets behaviour. Nat. Rev. Genet. 3, 114–123.

Carter, D.V., Lai, L., Park, K.W., Samuel, M., Lattimer, J.C., Jordan, K.R., Estes, D.M., Besch-Williford, C., Prather, R.S., 2002. Phenotyping of transgenic cloned piglets. Clon. Stem Cells 4, 131–145.

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. Behav. Brain Res. 31, 47–59.

Glantz, S.A., 2001. Primer of Biostatistics, 5th edition. McGraw-Hill, USA.

Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E.S., Golub, T.R., Jaenisch, R., 2002. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc. Natl. Acad. Sci. U.S.A. 99, 12889–12894.

Kornum, B.R., Thygesen, K.S., Nielsen, T.R., Knudsen, G.M., Lind, N.M., 2007. The effect of the inter-phase delay interval in the spontaneous object recognition test for pigs. Behav. Brain Res. 181, 210–217.

Kragh, P.M., Nielsen, A.L., Li, J., Du, Y., Lin, L., Schmidt, M., Bøgh, I.B., Holm, I.E., Jakobsen, J.E., Johansen, M.G., Purup, S., Bolund, L., Vajta, G., Jørgensen, A.L., 2009. Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res. 18, 545–558.

Moustgaard, A., Lind, N.M., Hemmingsen, R., Hansen, A.K., 2002. Spontaneous object recognition in the Gottingen minipig. Neural Plast. 9, 255–259.

Reik, W., 2007. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447, 425–432.

Ringman, J., 2005. What the study of persons at risk for familial Alzheimer's disease can tell us about the earliest stages of the disorder: a review. J. Geriatr. Psychiatry Neurol. 18, 228–233.

Russell, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Methuen & Co. Ltd, London.

Schmidt, M., Kragh, P.M., Li, J., Du, Y., Lin, L., Liu, Y., Bøgh, I.B., Winther, K.D., Vajta, G., Callesen, H., 2010. Pregnancies and piglets from large white sow recipients after two transfer methods of cloned and transgenic embryos of different pig breeds. Theriogenology 74, 1233–1240.

Søndergaard, L.V., Ladewig, J., Dagnæs-Hansen, F., Herskin, M.S., Holm, I.E., 2012. Object recognition as a measure of memory in 1–2 years old transgenic minipigs carrying the APPsw mutation for Alzheimer's disease. Transgenic Res., http://dx.doi.org/10.1007/s11248-012-9620-4.

Vajta, G., Gjerris, M., 2006. Science and technology of farm animal cloning: state of the art. Anim. Reprod Sci. 92, 211–230.