CSIRO PUBLISHING

Reproduction, Fertility and Development, 2016, **28**, 969–973
http://dx.doi.org/10.1071/RD14372

Impact of superovulation and mating on the wellbeing of juvenile and adult C57BL/6N mice

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Abstract. Superovulation of mice is routinely used to increase the number of obtainable ova per female. Because of the better outcome, prepubescent females are preferentially used. Here, we provide results of the impact of superovulation and mating on the wellbeing of juvenile compared with adult C57BL/6N mice. Two groups of mice (3–4 weeks vs 7–8 weeks old) were superovulated and mated. Observation of mating behaviour showed that reluctant adult females tended to fight the male's approach, whereas juveniles preferred to take flight. Faeces were collected daily for the analysis of stress hormones. There was no difference in the levels of glucocorticoid metabolites either between age groups or between treated animals and their controls. Histology after mating revealed intact vaginal mucosa without any detectable lesions in all animals regardless of age. In contrast to adults, almost all juveniles were synchronised in oestrus and produced significantly more ova. Taken together, our results reveal no increased welfare problem from using juvenile mice for superovulation and mating. Considering the higher yield of fertilisable oocytes and zygotes, it is advisable to use C57BL/6N prepubescent mice in order to reduce the number of donor females required.

Additional keywords: distress, mating behaviour, oestrous cycle, oestrus synchronisation, sexual maturation, vaginal histology.

Received 3 October 2014, accepted 12 November 2014, published online 17 December 2014

Introduction

Preimplantation mouse embryos are often required for biomedical research. All transgenic and assisted reproductive technologies applied in mice depend on oocytes and early embryos. For experimental and ethical reasons, it is desirable to harvest as many oocytes or embryos per donor as possible and consequently females are routinely superovulated by administration of exogenous hormones. Protocols for superovulation differ slightly between institutions with regard to hormone doses, the origin of the hormones and the exact time of injection during the daylight phase in the facility (Edgar *et al.* 1987; Legge and Sellens 1994; Johnson *et al.* 1996; Luo *et al.* 2011). The interval of 46–48 h between the pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) injection is undisputed. However, several publications reported that there are strong strain-dependent differences regarding the optimal age of the females for superovulation (Gates and Bozarth 1978; Hoogenkamp and Lewing 1982; Sugiyama *et al.* 1992; Ozgunen *et al.* 2001; Luo *et al.* 2011). For the widely used inbred strains C57BL/6J and C57BL/6N, the best results in terms of the number of harvested ova and embryos are achieved with juvenile females superovulated immediately after weaning and before puberty (Sugiyama *et al.* 1992; Byers *et al.* 2006; Luo *et al.* 2011). Furthermore, the quality of superovulated oocytes was not affected by the donor age, demonstrated by a similar percentage of fertilised oocytes (Luo

et al. 2011). Thus, the use of premature B6 females has become the standard procedure. However, reproduction before sexual maturity is unnatural and therefore the question arises if superovulation and mating of juvenile mice may induce pain and suffering in the animals. The recent discovery of a juvenile pheromone that exerts a powerful inhibitory effect on male mating behaviour suggests an inherent protective action of sexually immature mice (Ferrero *et al.* 2013). Therefore, the age-specific burden of the procedure should be considered for juveniles. In the present study we investigated and compared the possible distress induced by superovulation and subsequent mating to juvenile and adult females by observation of their mating behaviour, measurement of stress hormones and histology of the vaginal area.

Materials and methods

Animals (specific pathogen free) were housed in Makrolon cages (Tecniplast, Buguggiate, Italy) under standard laboratory conditions (room temperature 21 ± 1°C; relative humidity 40–55%; photoperiod 12 : 12 h, light cycle from 0600 to 1800 hours) and supplied with a standard breeding diet (V1126; Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water *ad libitum*. Mice were treated according to the institutional accredited guidelines. Experimental procedures were discussed and approved by the ethics committee of the University of Veterinary Medicine Vienna and granted by the national authority according to §26 of Law for Animal Experiments under licence number BMWF-68.205/0258-II/3b/2011.

Twenty-two juvenile (3–4 weeks, approx. 10 g) and 22 adult (7–8 weeks, approx. 16 g) C57BL/6N (Charles River Laboratories, Sulzfeld, Germany) females were separated at delivery into individual cages. For mating, 12 experienced adult males of the same strain were used.

Observation of mating behaviour and measurement of stress hormones

Mice of both age groups were delivered separately for three experimental rounds. The first two rounds consisted of 16 mice each (eight juveniles and eight adults) and were used for the assessment of the experimental burden by hormone levels and mating behaviour. The eight mice from each age group were randomly divided into experimental ($n = 5$) and control ($n = 3$) mice. Controls were not treated or mated and only used to evaluate baseline stress hormone levels. On Day 6 after arrival at 0900 hours, the experimental mice of both the young and the adult groups were treated for superovulation by an intraperitoneal (i.p.) injection of 5 IU PMSG (Folligon; Intervet, Vienna, Austria) and 48 h later 5 IU hCG (Chorulon; Intervet). Immediately after the second hormone application on Day 8, the females were mated with adult, experienced B6N males for 2 h in the male's cage. During mating, the mating behaviour of females and males was monitored. The following parameters were evaluated: male mounts, unsuccessful mating attempts, female flight and female repulsion. The last two parameters were defined by the female's (inferred) intent: 'female flight' was when the females ran laps along the cage wall while maintaining a few centimetres between the males and themselves while 'female repulsion' consisted of the females rearing on their back paws, raising their front paws in defiance and baring their teeth. However, if the male continued to advance, in all observed cases the female reverted to flight rather than actually fighting the male.

Concentrations of corticosterone metabolites were measured in the faeces of the animals in a non-invasive approach to assess the levels of stress that might result from superovulation and mating (Touma *et al.* 2003, 2004). As the peak of the excretion of corticosterone metabolites in faeces of mice is delayed by 8 to 10 h from the initial stress impulse (hormone application and mating; Touma *et al.* 2004), voided faeces were collected daily (1800–2100 hours) starting at the day of arrival (Day 1) to the day of mating (Day 8) and stored frozen (−20°C) until analysis. To avoid influences on the animal's circadian rhythm, a red light was used as the light source during collection. There were no care activities during the 8-day period of faeces collection to exclude confounding factors. For faeces collection, the mice were temporarily housed individually on paper towels without wood bedding. Samples were extracted with methanol and analysed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay as described in detail by Touma *et al.* (2003, 2004).

Superovulation, mating and vaginal histology

For the third round of experiments, 12 mice (six juveniles and six adults) were used to investigate both results of superovulation and vaginal histology. All mice from each age group were superovulated and mated overnight. The next morning, the females were killed by cervical dislocation and checked for a vaginal plug and swollen ampullae as an indication of successful mating and ovulation. After flushing of the dissected oviducts, fertilised, unfertilised and degenerated oocytes were counted for each donor.

The vagina and cervix of six juveniles and six adults were dissected immediately after isolation of the oviducts (24 h after hCG injection) and fixed *in situ* in buffered 4% formaldehyde solution for 48 h, then embedded in paraffin (Histocomp; Vogel, Giessen, Germany) using automated embedding equipment (Shandon Excelsior; Thermo Scientific, Waltham, MA, USA). Serial paraffin sections (thickness 3 μm) of vagina–cervix were cut longitudinally and stained with haematoxylin and eosin for routine morphological examination. Descriptive and comparative analysis of the sections was performed under light microscopy by a blinded investigator, taking into consideration inflammatory responses, integrity of treated tissues and organs and scarring.

Statistical analysis

Differences in mating behaviour between age groups and the number of fertilised, unfertilised and degenerated ova produced by juvenile and adult females after superovulation and mating were compared by *t*-test. Daily stress hormone levels of juvenile and adult mice measured non-invasively in the faeces were analysed by ANOVA with repeated-measurements followed by Tukey's honest significant difference (HSD) post hoc tests. Values were considered significantly different if $P \leq 0.05$.

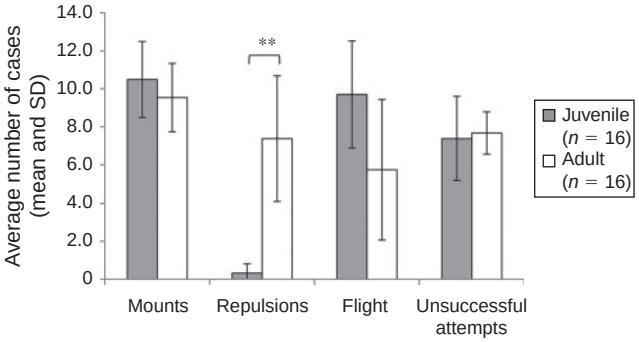

Fig. 1. Difference in mating behaviour between juvenile and adult C57BL/6N mice (**$P \leq 0.01$).

Results

Mating behaviour and levels of corticosterone metabolites

The number of attempted mounts by the males was almost equal in both groups. The reaction of females, however, revealed differences, mostly evident in the repulsive behaviour in adults ($P \leq 0.05$; Fig. 1) and in attempts to escape in juveniles.

No significant differences were observed in the levels of stress hormone metabolites between experimental groups and their controls or between juvenile and adult animals (Fig. 2). All measured animals experienced equal levels of distress, independent from hormone application or mating. Note that hormone treatment and mating were in the morning on Days 6 and 8, respectively, ~9 h before faeces collection in the evening.

Results of superovulation and histological analysis

Overall, significantly more ova were obtained from juvenile females compared with the adult group ($P \leq 0.05$; see Table S1, available as Supplementary Material to this paper), regardless of fertilisation. Although all animals were hormonally treated for superovulation, vaginal epithelial morphology was different between the two age groups: in all five successfully superovulated juveniles, a fully cornified stratified squamous epithelium was found, indicating that they were in oestrus after superovulation (Fig. 3). The sixth juvenile (JV3), from which no ova were obtained, was determined to be in proestrus. Even though all adults ovulated (suggesting that they were in oestrus), only three of the six had a vaginal histology that also corresponded to that cycle stage. Moreover, fertilised oocytes were only isolated from females AV4 and AV5, even though both were histologically not in oestrus (Fig. 3 and Table S1) and only female AV5 was plugged. In contrast to the adults, the results of superovulation of juveniles corresponded to their vaginal histology. Three of six treated juveniles were plugged (JV1, JV4, JV6) and all but one (JV3) had swollen ampullae and a high number of flushed ova. In four of the five successfully superovulated juvenile donors, fertilised ova were isolated (Table S1).

The vaginal histology revealed no indications of lesions or other injuries to the vaginal mucosa in any of the 12 examined mice.

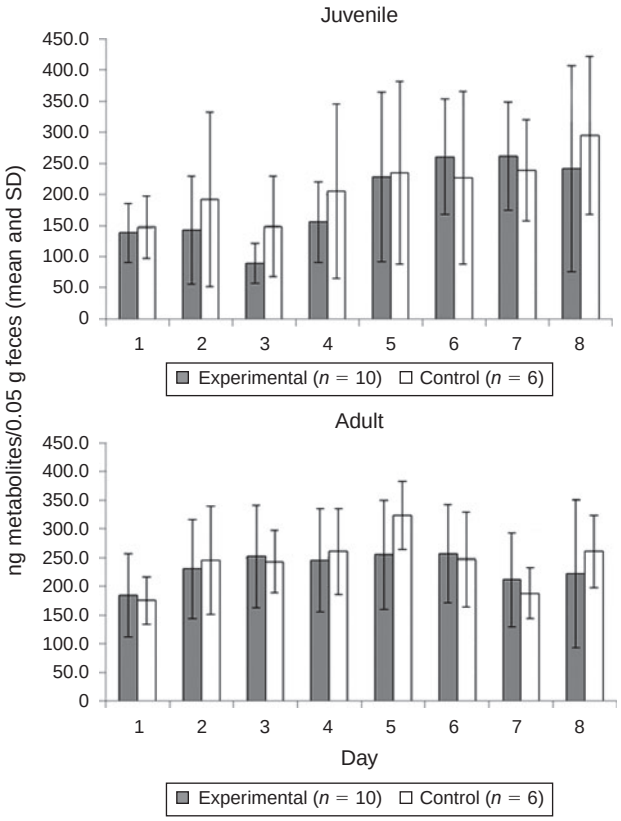

Fig. 2. Daily stress hormone levels of juvenile and adult C57BL/6N mice measured non-invasively in the faeces. Note the time points of possible stress impulses on Day 6 (PMSG injection) and Day 8 (hCG injection and mating).

Discussion

In this study, the effect of donor age in C57BL/6N mice was evaluated with regard to the distress of the females induced by the hormonal treatment and mating and the response to superovulation. We assessed the females' mating behaviour, stress hormone metabolites, genital lesions and vaginal epithelial morphology to determine the individual ovarian cycle. The number of unfertilised and fertilised oocytes per treated donor was counted and the results confirmed the general assumption of better results after superovulation of juvenile B6N mice (Table S1).

Many factors affect the outcome of superovulation treatment in mice, ranging from environment (Miyoshi *et al.* 1993), time schedule for injections and oocyte collection (Vergara *et al.* 1997), hormone doses (Edgar *et al.* 1987) and the age of donors related to the bodyweight (Gates and Bozarth 1978; Sugiyama *et al.* 1992; Ozgunen *et al.* 2001; Luo *et al.* 2011). Furthermore, the receptivity of the ovaries for exogenous hormones is genetically controlled (Spearow 1988; Spearow and Barkley 1999) and it was already shown more than 50 years ago that the outcome of superovulation is clearly age dependent (Zarrow and Wilson 1961). These observations led to the conclusion that every mouse strain has an optimal age for superovulation which is usually either 'premature'–'prepubertal' or 'mature'–'postpubertal'. For the widely used inbred strain C57BL/6J, the age at

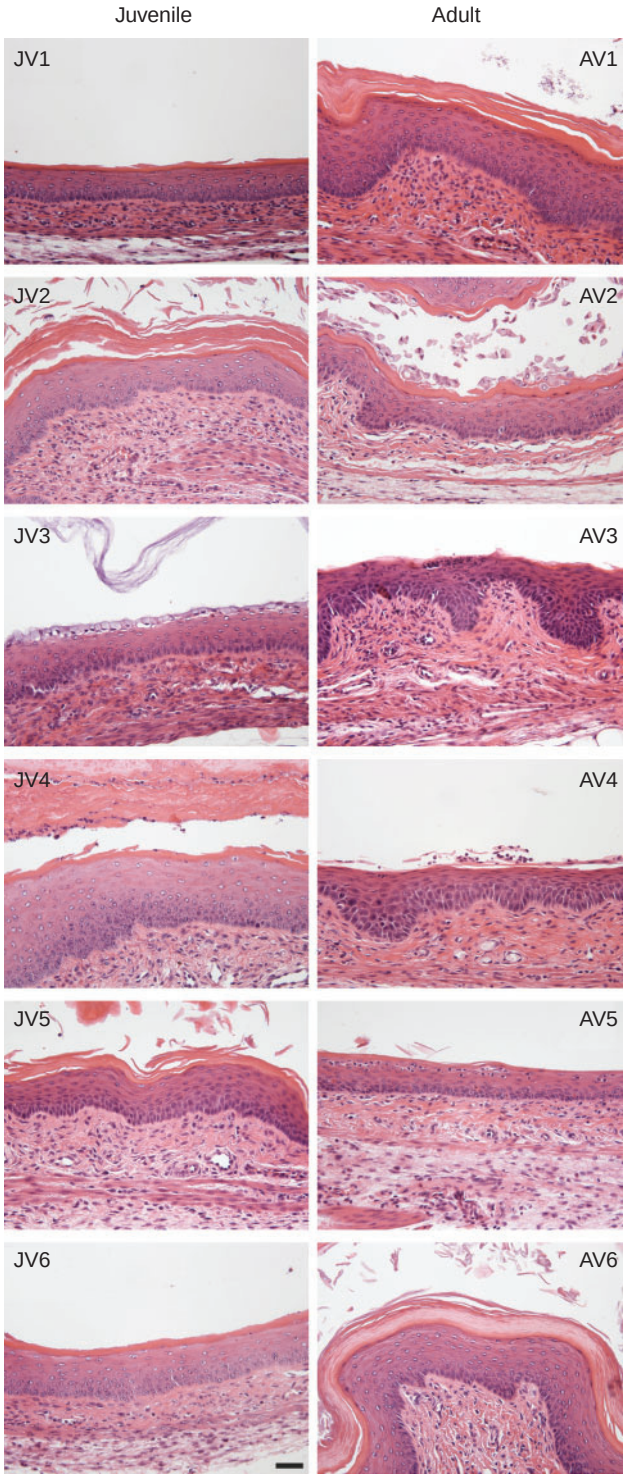

Fig. 3. Vaginal epithelium of juvenile (JV) and adult (AV) C57BL/6N mice 24 h after superovulation and mating. JV1, JV2, JV4, JV5 and JV6 are in oestrus, JV3 in proestrus. AV1, AV2 and AV6 are in oestrus, AV5 in metestrus, AV3 and AV4 in diestrus. Staining with haematoxylin & eosin (H&E). Scale bar = 50 µm.

vaginal opening indicating sexual maturation is ~34 days (Yuan *et al.* 2012) and the optimal donor age is suggested to be around 21 days (Sugiyama *et al.* 1992; Byers *et al.* 2006; Luo *et al.* 2011). Our results confirm these references insofar as premature superovulated donors produced significantly more oocytes (Table S1). However, is it justifiable in terms of the animal welfare to treat juvenile mice with hormones and mate them with a distinctly older and heavier male? To our knowledge, there are no data available about the impact of this approach on the wellbeing of the treated animals.

Especially for nulliparous female mice, the interaction with the male conspecific for mating is a completely new experience and C57BL/6N mice are described to have 'high' and 'goal-focussed' copulative behaviour (McGill 1962; Carola *et al.* 2008). The recently discovered secretion of a juvenile pheromone from the lacrimal gland that is released into tears of sexually immature mice (Ferrero *et al.* 2013) could potentially interfere with the mating of freshly weaned females by affecting male mating behaviour. The pheromone, termed exocrine-gland secreting peptide 22 (ESP22), inhibits sexual behaviour of adults towards young. Its expression is strongly increased in juveniles of both sexes and decreases promptly near puberty (Ferrero *et al.* 2013). However, it is not known if the secretion of ESP22 is possibly changed or suppressed by the artificial sexual maturation in superovulated juvenile females, which would circumvent this protection.

Our study revealed that juveniles and adults differ in their mating behaviour. Juvenile and adult females had different strategies to counteract the approach of the males before they were willing to mate (Fig. 1). The adult group seemed to balance their counterattacks with flight reflexes and choose between fight and flight equally, whereas the juvenile group had to resort to escaping almost every time the male approached uninvited. After willingness for mating was established, the number of successful mounts and unsuccessful mating attempts was comparable between both age groups, suggesting that the male's mating behaviour was not inhibited or changed in the juvenile female group.

For the assessment of distress induced by hormone treatment and mating we compared levels of faecal corticosterone metabolites between treated and untreated juvenile and adult females (Fig. 2). The observation of the frequently used self-rewarding behaviours like nest building or burrowing as indication for pain and suffering are not very applicable for this approach because the animals will usually be killed soon after hormone treatment and mating. Despite the observed differences in mating behaviour between the adult and juvenile B6N females, there was surprisingly no increase in the levels of stress hormone metabolites in any treatment group at any time point. The non-invasive measurement of corticosterone metabolites in the faeces has been proven to be a good indicator of distress in laboratory mice (Touma *et al.* 2003; Gurfein *et al.* 2012). However, compared with previous data, the level of corticosterone metabolites was generally increased in all mice of this study including controls, possibly due to the impact of not allowing the mice time to adapt to the new environmental and experimental conditions after delivery. Starting the experiment immediately after arriving was necessary to ensure that juvenile

females were still prepubescent at the time point of superovulation. In addition, the strong diurnal changes of corticosterone concentrations reach the maximal values in the first third of the dark phase, i.e. the period used for faeces collection of this study (Touma *et al.* 2004). We can also not exclude a strain-specific impact on the results of corticosterone metabolites, although C57BL/6J mice are known for a lower sensitivity to subchronic mild stress and for a lower level of anxiety-like behaviour than other inbred strains (Ducottet and Belzung 2005; An *et al.* 2011).

The histology of reproductive organs showed no lesions on the vaginal mucosa of either juvenile or adult females. However, the expected synchronised induction of oestrus after hormonal treatment was only seen in the juvenile group. Although a low level of ovulation was induced in all six treated adults, females of this group varied in the cycle phases demonstrated by the individual characteristics of the vaginal epithelium. Since cytological changes of the uterine mucosa reflect the underlying endocrine events of the respective cycle phase, the observed discrepancy between adult females after superovulation indicate an interfering impact of the endogenous ovarian cycle on the hormonal treatment (Fig. 3).

In summary, our results reveal a difference in the mating behaviour but no evidence for increased distress or injuries when using juvenile C57BL/6N mice for superovulation and mating. In contrast to juveniles, the vaginal histology indicated an incomplete synchronisation and oestrus induction in hormone-treated adult mice. Considering the higher yield of oocytes and zygotes, it is advisable to use juvenile mice of this strain in order to reduce the number of required donor females.

Acknowledgements

We acknowledge Dr Alexander Tichy, Statistical Ambulance, University of Veterinary Medicine Vienna for help in statistical analysis and the excellent technical assistance of Edith Klobetz-Rassam for analysis of corticosterone metabolites. Special thanks go to Dalma Batkay and Tien Yin Yau for editing the manuscript. The work was supported by the FP7/ INFRAFRONTIER-I3 (Grant Agreement number: 312325) project to T. R. and by the University of Veterinary Medicine Vienna.

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Supplementary Material: *Reproduction, Fertility and Development*, 2016, 28(7), 969–973.

Supplementary Material

Impact of superovulation and mating on the wellbeing of juvenile and adult C57BL/6N mice

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Table S1. Results of the plug and ampulla control after superovulation and mating

Number of fertilised, unfertilised and degenerated ova produced by juvenile and adult females (*$P \leq 0.05$)

Juvenile	**Plug**	**Ampulla**	**Ova**		
			Fertilised	**Unfertilised**	**Degenerated**
JV1	+	Swollen	23	3	0
JV2	-	Swollen	0	57	0
JV3	-	Not swollen	0	1	0
JV4	+	Swollen	38	5	0
JV5	-	Swollen	31	35	0
JV6	+	Swollen	22	20	0
Mean			19*	20.17	0
SD			15.82	22.22	0
Adult	**Plug**	**Ampulla**	**Ova**		
			Fertilised	**Unfertilised**	**Degenerated**
AV1	-	Swollen	0	8	0
AV2	-	Swollen	0	11	6
AV3	-	Swollen	0	12	0
AV4	-	Swollen	11	3	4
AV5	+	Not swollen	7	4	0
AV6	-	Swollen	0	12	0
Mean			3*	8.33	1.67
SD			4.82	4.03	2.65